STUDIES ON THE SUITABILITY OF *Jatropha curcas* KERNEL MEAL AS AN ALTERNATIVE PROTEIN SOURCE IN DIETS FOR CARP (*Cyprinus carpio*) AND TILAPIA (*Oreochromis niloticus*)

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by

Carsten Alexander Krome (MSc)

July 2014

UNIVERSITY OF STIRLING

School of Natural Sciences

Institute of Aquaculture

Declaration

I hereby declare that this thesis has been composed entirely by myself and has not been submitted for any other higher degree or qualification. All sources of information have been suitably acknowledged in the text.

Name: Carsten Alexander Krome

Sign:

Date:

Primary supervisor:

Sign:

Date:

Acknowledgements

It is my pleasure to thank all those people who made this project possible.

I would like to express my gratitude towards my supervisors Prof. Dr. Ulfert Focken and Dr. Kim Jauncey.

Für meine Eltern, Hannelore und Hartmut, für ihre grenzenlose Unterstützung.

Abstract

Aquaculture production is increasing annualy and wild fisheries for fishmeal production remain stagnant. As a consequence, extensive research has been deployed to reduce dietary fishmeal inclusion in feeds of farmed species. Usage of alternative protein sources derived from plants continues to increase with the most popular sources being oilseeds, legumes and cereal grains. The downside of these sources is that most of them could directly be used for human consumption arising legitimate criticism from voices referring to countries where protein shortages lead to malnutrition among the population.

Jatropha curcas is a tropical oilseed with upcoming popularity for sustainable fuel sourcing. The plant is thought to thrive in semi-arid and arid areas, not just producing oil, but at the same time reclaiming previously eroded land for the local population. For these reasons, annual cultivation of *Jatropha curcas* is thought to rise over the next decades.

After oil is extracted from the seed, the remaining press cake, is currently used as a fertilizer or energy source. This is mostly due to toxic phorbol esters that until recently limited any nutritional applications. In 2011, a method to detoxify *Jatropha* press cake was developed and paved the way for nutritional research on the resulting detoxified *Jatropha curcas* kernel meal (JKM) to be launched. JKM offers very high protein content with a balanced amino acid composition suggesting opportunities for usage as a feedstuff in aquaculture diets. JKM further has higher mineral content than comparable oilseeds. However, potential anti-nutritional factors (ANFs) present in the meal could lead to impairment of nutrient availability or other adverse effects. Previous research has already started to evaluate JKM as a protein source for a variety of aquaculture species. This thesis attempts to further identify the potential of JKM as a protein source and assess the effects of JKM on the development of two

model cultured teleost species, common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*), as they represent two of the most farmed species, particularly in the tropics.

The work comprises growth trials on both species using fishmeal as a subject of replacement in solely fishmeal protein-based diets and on more practical fishmeal/plant protein-based diets. It engages with the effects of phytate, a prominent component of JKM with potential anti-nutritional attributes and commercially available phytase feed supplements to diminish these attributes, as well as oxalate, another plant-specific component with high concentrations in JKM and with limited attention in aquaculture nutrition research.

Carp and tilapia showed varying results with different inclusion levels of JKM. For carp 50% fishmeal replacement was possible without losses in growth in diets where fishmeal was the only bulk protein source (Chapter 3.1), Tilapia showed slightly worse growth at a 30% replacement level (Chapter 3.2). A steep decline in growth could be observed when replacing 100% fishmeal with JKM in carp (Chapter 3.1), while tilapia showed no difference at that level compared to 30% replacement (Chapter 3.2). In practical diets, 100% of fishmeal could be replaced by JKM without any adverse effects on growth of carp (Chapter 3.3), while tilapia showed a slight, but significant linear negative correlation with higher inclusion levels of JKM (Chapter 3.4).

Phytase addition in tilapia feeds was identified as having no obvious impact on growth in JKM based diets where enough available phosphorus was provided through mineral supplementation (Chapter 4.1). In JKM based diets where available phosphate was not added, phytase addition showed a tendency to increase growth and significantly increased mineral retention and decreased phosphorus effluent contamination (Chapter 4.2). Phytase application through pre-incubating JKM along

with citric acid exerted a positive effect of growth on carp when fishmeal protein was replaced by 50% (Chapter 3.1). Phytase was further shown to completely hydrolyze phytate *in vitro*; however, incomplete hydrolysis was observed *in vivo* in tilapia (Chapter 4.3).

Dietary soluble oxalate added to fishmeal based diets for carp showed better growth parameters, nutrient and mineral retention at inclusion levels 1.5% and higher (Chapter 5.1). For tilapia, a trial could demonstrate adverse effects of oxalate on potassium, calcium, manganese and zinc digestibilities, in this case without negative effects on growth (Chapter 5.2). For both, carp and tilapia, an impact of oxalate on lipid metabolism was evident, lowering body lipid content and blood cholesterol in inclusion levels from 1.5% or higher.

JKM can become a valuable alternative to present dietary protein sources in aquaculture feeds. The nutritional attributes of JKM need further research, especially longer-term testing in a commercial scenario and application in commercially produced feeds. Results of this thesis pose a useful addition to previous research and can be referred to for realizing these next steps.

Table of Contents

Declaration II
Acknowledgements III
Abstract IV
Table of Contents VII
List of Tables XI
List of Figures XVI
List of Abbreviations XVII

1 General Introduction 20
1.1 Plant-based feed ingredients in aquaculture diets 20
1.2 The plant *Jatropha curcas* 23
1.2.1 General information 23
1.2.2 Applications of *Jatropha curcas* in the biofuel industry 24
1.3 Characteristics of *Jatropha curcas* kernel meal (JKM) 26
1.3.1 Crude protein and essential amino acid content 26
1.3.2 Fat and ash content 28
1.3.3 Anti-nutritional factors (ANFs) 32
1.3.4 Detoxification of *Jatropha curcas* press cake 43
1.4 Previous work on JKM in fish diets 44
1.5 Aims and scopes of the project 46
1.6 References 48
2 General Material and Methods 58
2.1 Experimental design and set-up 58
2.1.1 Recirculation systems 58
2.1.2 Experimental design 59
2.1.3 Feeding schedule 60
2.2 Production of experimental diets 61
2.3 Pre- and post-experimental activities 61

2.4 Chemical and biochemical analyses 63
2.4.1 Dry matter 63
2.4.2 Lipid 63
2.4.3 Ash 64
2.4.4 Protein 65
2.4.5 Energy 66
2.4.6 Minerals 66
2.4.7 Digestibility analysis 67
2.4.8 Blood plasma analysis 67
2.4.9 Phytate 68
2.4.10 Calculations and equations 68
2.4.11 Statistical analysis 70
2.5 References 71

3 Inclusion of *Jatropha curcas* Kernel Meal in Fishmeal-based and Practical Diets for Tilapia and Carp 73

3.1 Phytase-incubated *Jatrophas curcas* kernel meal in diets for carp (*Cyprinus carpio*) 73
3.1.1 Introduction 74
3.1.2 Material and methods 74
3.1.3 Results 77
3.1.4 Discussion 83
3.1.5 References 87
3.2 Effect of Replacing Different Levels of Dietary Fish Meal with *Jatropha curcas* Kernel Meal on the development of Nile Tilapia (*Oreochromis niloticus*). 90
3.2.1 Introduction 91
3.2.2 Material and methods 91
3.2.3 Results 94
3.2.4 Discussion 98
3.2.5 References 101

3.3 Influence of a sodium formate / formic acid mixture on growth of juvenile common carp (*Cyprinus carpio*) fed different fishmeal replacement levels of detoxified *Jatropha curcas* kernel meal in practical, mixed diets. 105

3.3.1 Introduction 106

3.3.2 Material and Methods 107

3.3.3 Results 109

3.3.4 Discussion 118

3.3.5 References 123

3.4 *Jatropha curcas* kernel meal as a replacement for fish meal in practical Nile tilapia (*Oreochromis niloticus*, Linaeus, 1758) feeds 128

3.4.1 Introduction 129

3.4.2 Material and Methods 129

3.4.3 Results 132

3.4.4 Discussion 136

3.4.5 References 141

4 The Role of Phytate as an Anti-nutritional Factor in JKM-based Diets for Tilapia 145

4.1 Testing two different phytases in *Jatrophas curcas* kernel meal based diets for Nile tilapia, *Oreochromis niloticus* 145

4.1.1 Introduction 146

4.1.2 Material and methods 146

4.1.3 Results 149

4.1.4 Discussion 152

4.1.5 References 155

4.2 Influence of phytase on growth and mineral retention of tilapia fed non-phosphate supplemented *Jatropha curcas* kernel meal-based diets 160

4.2.1 Introduction 161

4.2.2 Material and methods 161

4.2.3 Results 163

4.2.4 Discussion 167

4.2.5 References 172

4.3 Phytate analysis, *in vitro* and *in vivo* phytase application in *Jatropha curcas* kernel meal 177

4.3.1 Introduction 178

4.3.2 Material and methods 179

4.3.3 Results 182

4.3.4 Discussion 187

4.3.5 References 190

5 Dietary Oxalate as an Anti-nutritional Factor in Basic Diets for Carp and Tilapia 194

5.1 Effect of dietary sodium-oxalate on growth, body composition, nutrient retention and blood parameters of common carp (*Cyprinus carpio*) 194

5.1.1 Introduction 195

5.1.2 Material and methods 195

5.1.3 Results 198

5.1.4 Discussion 200

5.1.5 References 206

5.2 Dietary oxalate reduces potassium, calcium, manganese and zinc digestibilitiy in tilapia (*Oreochromis niloticus*) 210

5.2.1 Introduction 211

5.2.2 Material and methods 211

5.2.3 Results 215

5.2.4 Discussion 219

5.2.5 References 224

6 General Discussion 227

6.1 Growth of carp and tilapia fed diets containing different levels of JKM ... 228

6.2 Body composition of carp and tilapia with different levels of JKM in the diet 230

6.2.1 Protein 230

6.2.2 Lipid 231

6.2.3 Ash 233

6.3 Influence of phytate and application of phytase in JKM-based diets 233

6.4 Influence of dietary oxalate on growth and body composition of common carp and tilapia 236

6.5 Aims and scopes of the project 238

6.6 References 239

List of Tables

Table 1.1.1 Global production of cereals, oilseeds and pulses as potential feed ingredients for aquaculture diets in 2009 22

Table 1.1.2 Global production of oilseed meals in 2009 22

Table 1.2.1 Different oil-seed species producing unedible oil for biofuel in India. ... 25

Table 1.3.1 Comparison of nutrients and essential amino acids of different plant-based feedstuffs to fishmeal 30

Table 1.3.2 Comparison of two essential and non-essential amino acid compositions of two different batches of JKM as well as essential amino acid requirements of carp and tilapia 31

Table 1.3.3 Total mineral-content of JKM compared to fishmeal, soybean meal and rice bran 32

Table 1.3.4 Anti-nutritional factors in toxic type of *Jatropha curcas* kernel meal (untreated) 33

Table 1.3.5 Soluble oxalate contents of selected fodder crops (% DM). 41

Table 3.1.1 Composition of experimental diets with 50% and 100% fishmeal protein replacement through JKM with and without phytase incubation fed to common carp for 8 weeks 76

Table 3.1.2 Proximate composition of experimental diets fed to common carp for 8 weeks. 76
Table 3.1.3 Phytate contents of experimental diets with and without phytase-incubation fed to common carp for 8 weeks. 78
Table 3.1.4 Whole body chemical composition common carp at the start and after fed experimental diets for 8 weeks. 79
Table 3.1.5 Body mass gain of experimental fish fed different levels of JKM with and without incubation for 8 weeks. 79
Table 3.1.6 Growth parameters and nutrient utilization of experimental fish fed different levels of JKM with and without phytase incubation for 8 weeks. 81
Table 3.2.1 Composition of experimental diets containing different levels of JKM fed to Nile tilapia for 8 weeks. 93
Table 3.2.2 Proximate composition of experimental diets diets containing different levels of JKM and fed to Nile tilapia for 8 weeks. 93
Table 3.2.3 Water parameters measured during the experimental duration. 94
Table 3.2.4 Growth parameters and nutrient retention of Nile tilapia fed different levels of JKM for 8 weeks. 97
Table 3.2.5 Proximate composition and gross energy of whole body of Nile tilapia fed different levels of JKM for 8 weeks. 98
Table 3.3.1 Nutrient (% dry weight) and amino acid (g / 100g protein) composition of JKM. 110
Table 3.3.2 Nutrient (%) and essential amino acid (EAA, g / 100g protein) composition of feedstuffs used in the experimental diets. 111
Table 3.3.3 Composition of experimental diets containing different replacement levels of fishmeal with JKM with and without NDF. 112
Table 3.3.4 Growth parameters of common carp fed experimental diets containing different levels of JKM with and without NDF for 8 weeks. 114

Table 3.3.5 Body composition of common carp before and after fed different levels of JKM with and without NDF for 8 weeks. 115

Table 3.3.6 Dry matter and protein digestibility of common carp fed different levels of JKM with and without NDF for 8 weeks. 116

Table 3.3.7 Nutrient and mineral retention of common carp fed different contents of JKM with and without NDF for 8 weeks. 117

Table 3.3.8 Blood plasma parameters of common carp fed different contents of JKM with and without NDF for 8 weeks. 117

Table 3.4.1 Nutrient (g / kg) and amino acid (g / kg protein) composition of detoxified JKM in comparison to fishmeal and nutrient and essential amino acid composition of other applied feedstuffs.. 130

Table 3.4.2 Composition and proximate analysis of diets containing different levels of JKM fed to Nile tilapia for 8 weeks. 131

Table 3.4.3 Survival of experimental fish fed diets containing different levels of JKM for 8 weeks. 133

Table 3.4.4 Body composition of experimental fish fed different levels of JKM for 8 weeks. 135

Table 3.4.5 Growth performance parameters and nutrient utilization of fish fed different levels of JKM for 8 weeks. 136

Table 3.4.6 Effect of feeding different levels of JKM on apparent digestibility coefficients of dry matter, protein and energy in Nile tilapia. 136

Table 4.1.1 Composition and proximate analysis of diets fed to Nile tilapia for 8 weeks. 148

Table 4.1.2 Body composition of Nile tilapia fed different experimental diets for 8 weeks. 150

Table 4.1.3 Feed utilization parameters of Nile tilapia fed experimental diets for 8 weeks. 151

Table 4.2.1 Composition of diets containing no addition, phytase and / or phosphate addition fed to Nile tilapia for 8 weeks... 163
Table 4.2.2 Body mass gain of Nile tilapia fed JKM based diets with and without phytase and / or phosphate addition.. 164
Table 4.2.3 Feed utilization parameters of Nile tilapia fed JKM based diets with and without phytase and / or phosphate addition for 8 weeks.......................... 164
Table 4.2.4 Body composition of of Nile tilapia fed JKM based diets with and without phytase and / or phosphate addition for 8 weeks..................................... 166
Table 4.2.5 Retention of selected minerals of Nile tilapia fed JKM based diets with and without phytase and / or phosphate addition for 8 weeks.................. 167
Table 4.2.6 Nitrogen and phosphorus load of effluent water from tilapia fed JKM based diets with and without phytase and / or phosphate for 8 weeks...... 167
Table 4.3.1 Composition of the two experimental diets with and without phytase addition fed to Nile tilapia to obtain faeces for inositol phosphate and mineral analysis. 183
Table 4.3.2 Anion content of JKM and soybean meal acid extract. 184
Table 4.3.3 IP-values of acid extract of JKM with no incubation (“JKM”) or after *in vitro* treatment with (“Phytase”) and without phytase (“Control”). 186
Table 4.3.4 IP-concentration in faeces of Nile tilapia fed diets with (“Phytase”) and without phytase (“Control”). 186
Table 4.3.5 Faeces mineral analysis of Nile tilapia fed JKM-based diets with (“Phytase”) and without phytase (“Control”). 187
Table 5.1.1 Composition of experimental diets containing 0%, 0.5%, 1.5% and 2.5% of oxalate fed to common carp for 8 weeks... 196
Table 5.1.2 Body mass gain of common carp fed different concentrations of soluble oxalate for 8 weeks... 198

Table 5.1.3 Nutrient utilization of common carp fed different concentrations of oxalate for 8 weeks.. 199

Table 5.1.4 Body nutrient and mineral composition of common carp fed different concentrations of oxalate for 8 weeks... 199

Table 5.1.5 Faeces mineral composition of common carp fed different concentrations of oxalate for 8 weeks... 200

Table 5.1.6 Mineral retention of selected minerals of common carp fed different concentration of oxalate for 8 weeks. 201

Table 5.1.7 Selected blood plasma parameters of common carp fed different level of oxalate for 8 weeks.. 201

Table 5.2.1 Compositions and proximate analysis of experimental diets containing different levels of oxalate and fed to Nile tilapia in the growth and the histology trial. 214

Table 5.2.2 Survival of Nile tilapia fed 0%, 0.5%, 1.5% and 2.5% of oxalate for 6 weeks 216

Table 5.2.3 Growth parameters of Nile tilapia fed different dietary oxalate concentrations for 6 weeks. 216

Table 5.2.4 Apparent dry matter, protein and mineral digestibilities of Nile tilapia fed different dietary oxalate levels for 6 weeks. 217

Table 5.2.5 Body nutrient and mineral composition of Nile tilapia fed different dietary oxalate levels for 6 weeks.. 218

Table 5.2.6 Selected blood plasma values of Nile tilapia of the histology trial fed experimental diets for 4 weeks. 219

Table 6.2.1 Body composition of carp and tilapia in accordance to diet type and fishmeal replacement level of JKM – comparison across trials. 231

List of Figures

Figure 1.1.1 Structure of thesis XIX

Figure 1.3.1 Flow chart of processing of *Jatropha curcas* seeds with relative yields of oil and de-oiled press cake. 27

Figure 1.3.2 The molecular structure of phytate. 37

Figure 1.3.3 The molecular structure of oxalate. 42

Figure 2.1.1 Diagram of recirculation system used for experiments. 59

Figure 3.1.1 Growth curves of fish fed different levels of JKM with and without phytase incubation for 8 weeks.. 80

Figure 3.1.2 FCR progression of fish fed 50% of protein through JKM with and without phytase incubation. 82

Figure 3.1.3 SGR progression of fish fed 50% of protein through JKM with and without phytase incubation. 82

Figure 3.2.1 Body mass development of tilapia (*Oreochromis niloticus*) fed experimental diets based on *Jatropha curcas* kernel meal for 8 weeks....... 95

Figure 3.2.2 Development of feed conversion ratio of tilapia (*Oreochromis niloticus*) fed experimental diets based on *Jatropha curcas* kernel meal over the course of the experiment. 96

Figure 3.4.1 Body weight development of Nile tilapia fed different levels of JKM for 8 weeks. 133

Figure 4.1.1 Growth of *Oreochromis niloticus* fed different experimental diets. 150

Figure 4.3.1 Phytate content of JKM measured with different colorimetric methods. 184

Figure 4.3.2 Interference of selected anions of JKM-extract on absorption of the Wade-reagent. From up left to down right: Phytate, oxalate, citrate,

phosphate. Malate and sulfate showed no interference with the Wade reagent (not shown)... 185

Figure 5.2.1 Kidney cross-sections of Nile tilapia fed 0% oxalate (left) and 2.5% oxalate (right) for 4 weeks. 219

List of Abbreviations

AA	Amino acid
ADC	Apparent digestibility coefficient
ADF	Acid detergent fibre
ADL	Acid detergent lignin
BMG	Body mass gain
CA	Crude ash
CL	Crude lipid
CP	Crude protein
DM	Dry matter
EAA	Essential amino acid
EPV	Energy productive value
FCR	Feed conversion ratio
FM	Fishmeal
FW	Final body weight
HPIC	High pressure ion chromatography
ICP	Inductively Coupled Plasma
IP	Inositol phosphate
IP1-IP6	Inositol mono- to hexa phosphate
IW	Initial body weight
JKM	*Jatropha curcas* kernel meal
LPV	Lipid productive value
MGR	Metabolic growth rate
MS	Mass spectrometer

MT	Metric ton
MT	Million tons
NDF	Neutral detergent fibre
NDF	Commercial formic acid / formate mix (ADDCON GmbH, Germany)
NFE	Nitrogen free extract
NSP	Non-starch polysaccharides
PER	Protein efficiency ratio
PPV	Protein productive value
SBM	Soybean meal
SGR	Specific growth rate
SPC	Soy protein concentrate
TI	Trypsin inhibitor
TMT	Thousand metric tons
U	Unit quantifying activity of enzymes

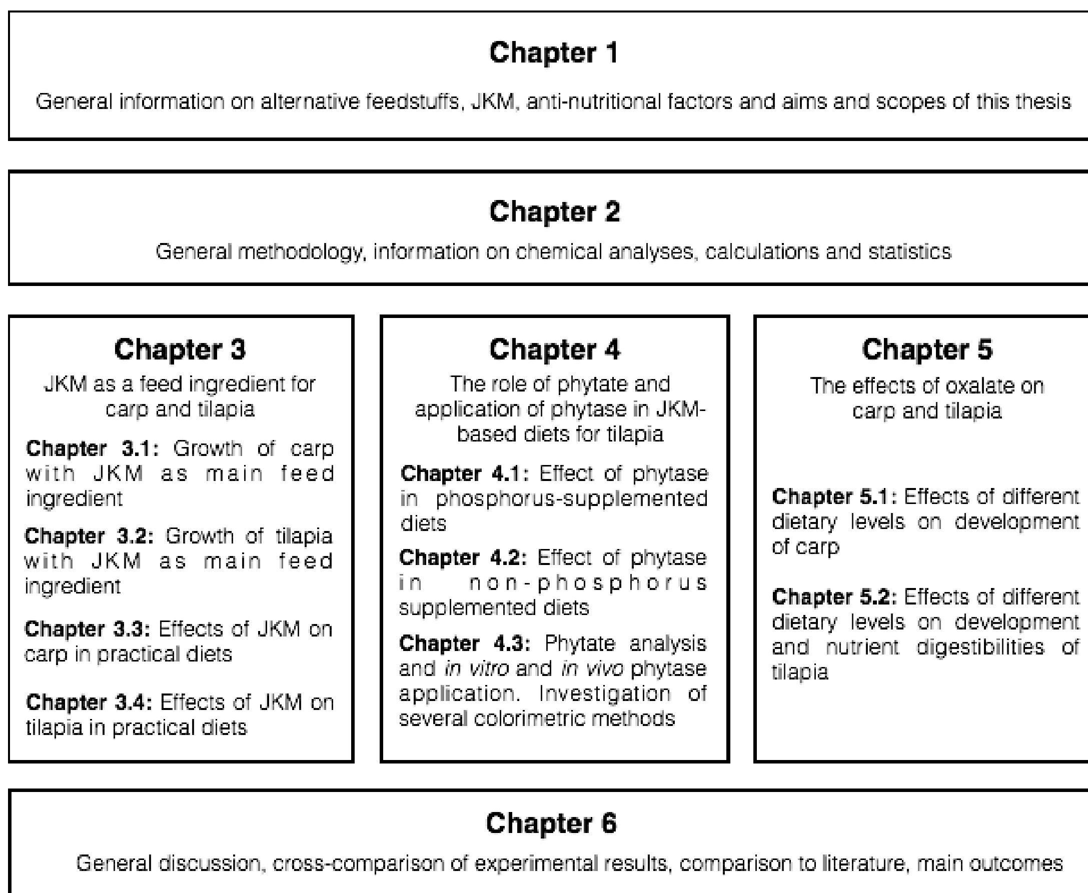


Figure 1.1.1 Structure of thesis

1 General Introduction

1.1 Plant-based feed ingredients in aquaculture diets

Global fish meal production averaged around 6.5 million tons over the last 20 years (Hardy, 2010). In 1988, more than 90% of the annual production was consumed by poultry and pork feed production. Its relative use in aquaculture has continuously increased since, predominantly due to the fast expansion of the whole sector. By 2010, 56% of global fishmeal production was consumed by the aquaculture sector (Olsen & Hasan, 2012). In 2006, marine fish (excluding salmon species) and shrimp production used about 663.1 thousand metric tons (TMT) and 989.7 TMT of fish-meal, respectively. Tilapia and carp feeds used 192.2 TMT and 423.3 TMT (Tacon & Metian, 2008). An estimate of net fish-in fish-out ratio in the year 2006 was 0.7, meaning that for each 0.7 tons of fish (wet weight) 1 ton of fish (wet weight) was produced. This figure is highly species dependent with salmon species having a ratio of 4.9, while tilapia and carp have ratios of 0.4 and 0.2 respectively (Muir, 2013).

Fishmeal production is unlikely to increase beyond current levels and as the aqua-culture sector experienced steady annual growth between 5% and 8% in the last decade (FAO, 2013), the need for alternative protein sources for aquaculture feeds is evident. Potential plant-based feedstuffs are numerously available (Table 1.1.1). Oil-seeds and meals produced out of oil-seeds (Table 1.1.2) represent the second biggest category of crops after cereals.

Since 1995, there have been steady improvements in decreasing the percentage of fishmeal in commercial diets, i.e. the fish-in fish-out ratio has dropped on average about 50% since then (Tacon & Metian, 2008). Fish species such as carp and tilap-

ia, which incorporate plant feedstuffs in their natural diet, have better capabilities to effectively digest these components than carnivorous species. While fishmeal is still the most effective feedstuff, it has been achieved in carp and tilapia to replace fishmeal nearly completely from the diet (Tacon & Metian, 2008). Nonetheless, there are various difficulties when replacing fishmeal with plant-based feed ingredients. Essential amino acid (EAA) composition is mostly different to the one of fishmeal and to the optimal requirements of the cultured species and this may limit growth (Ayadi et al., 2012). Further, plant-based ingredients contain components that can adversely affect nutrient availability or health of fish. They are summarized as antinutritional factors and comprise a large variety of substances, some of which resist heat treatment, the most commonly applied method for destroying them (Francis et al., 2001). Another potential problem with increasing replacements of fishmeal by plant meal may be unknown compounds available in fishmeal, but not in plant meal, which are beneficial to the fish. While the available amino acids, carbohydrates, lipids, minerals and vitamins are well-documented, there are more components in fish meal and it is likely that these have an influence on its performance. These components may include trace- and ultra-trace metals and amines, such as taurine or steroids (Hardy, 2010).

Plant-based ingredients are not necessarily more cost-effective than fishmeal. The price of fishmeal is continuously increasing, however, the less competitive fishmeal becomes, the more competitive become plant-based feedstuffs and consequently prices increase. Prices for corn, wheat and soy more than doubled over the period from 2007 to 2008 (Hardy, 2010).

In addition, most plant-based feed stuffs are competing with human nutritional demands. *Jatropha curcas* produces inedible oil and meal and is thought to thrive on marginal land unsuitable for production of food plants (Becker et al., 2013).

Table 1.1.1 Global production of cereals, oilseeds and pulses as potential feed ingredients for aquaculture diets in 2009

Cereals	**MT**
Maize	817
Wheat	682
Rice paddy	679
Barley	150
Sorghum	62
Others	99
Total	2489
Oilseeds	
Soybean	222
Rapeseed	62
Cottonseed	41
Groundnuts	36
Sunflower seed	32
Palm kernel	12
Others	9
Total	414
Pulses	
Peas	10.5
Lupin	0.9
Total	11.4

Values in million tons
FAO, 2011

Table 1.1.2 Global production of oilseed meals in 2009

Meal	**Amount**
Soybean meal	151.5
Rapeseed	30.8
Cottonseed	14.4
Sunflower seed	12.6
Palm kernel	6.2
Peanut	6.0
Copra	1.9
Jatropha[1]	1.14

Values is million tons
FAO, 2011
[1]Assuming 2 tons / ha seed yield, 900 000 ha plantation area and 35% seed oil content (Borman et al., 2013)

1.2 The plant *Jatropha curcas*

1.2.1 General information

Jatropha curcas or “purging nut” is a member of the family *Euphorbiaceae*, with around 240 genera. The genus *Jatropha* has between 165 and 175 species. The name “*Jatropha*” is derived from the Greek words “iatros” (doctor) and “trophe” (nutrition) and points out the medical applications of some species as well as the edible root tuber of the manioc/tapioca plant, which was previously placed to the same genus (Kumar & Sharma, 2008).

Jatropha curcas is diploid with 2n = 22 chromosomes. The genus is mostly monoecious, seldom dioecious, meaning female and male sex of the flower are separated, but on the same plant, turning them into hermaphrodites. The terminal florescence possesses a specific alignment. In the middle of the florescence is one or several female flowers and these are surrounded by male flowers. All flowers own 5 sepal and 5 petal leaves. Male flowers own 5 stamens, while female flowers posses 2-3 carpelae that are grown together to an ovary. Pollination is usually conducted by insects. The plants can fertilize themselves; however, through anthesis at different times, cross-pollination is encouraged. Capsules are developed, which burst when ripe and hurl the oil-containing seed several meters in the air (Makkar & Becker, 2009).

The genus *Jatropha* originated in tropical America around 70 million years ago, but has spread throughout the world. It is now systematically cultivated as a biofuel source. The plant is a shrub or small tree and can reach various sizes up to 12 m when solitary (Makkar & Becker, 2009). Of the two genotypes of *Jatropha curcas*, one is toxic and the other one is not. The non-toxic genotype is found only in Mexico.

Jatropha is a hardy plant that can sustain temperatures over 40°C and as low as light frost. It has been found to grow in areas with soils of very low nutrient value. Specifically phosphor deficiency seems to be not relevant to *Jatropha*. It has been reported that *Jatropha* can thrive in areas where there is no rain for two to three years. On the other hand, *Jatropha* can also tolerate humid conditions with high rain fall. However, flooding is not endured (Makkar & Becker, 2009).

1.2.2 Applications of *Jatropha curcas* in the biofuel industry

While the major part of all energy consumed originates from petroleum, natural gas and coal, renewable energy sources are on the ascent. In terms of fuel, the supplementation of fuel based on mineral oil with sustainably sourced biofuel has been on the rise since the first introduction of commercial biodiesel by the National Soy Diesel Development Board in the USA in 1992 (Koh & Ghazi, 2011). Global annual biodiesel production is projected to reach 35.3 MT by 2020 (OECD-FAO, 2011). However, production of biofuel from edible oil sources has been criticized for the fact that it could be better used in the nourishment of humans, especially in countries where local, edible oil production does not meet local demand. An example is India, where oil seed plantations for bio fuel production are frequent, but between 2007 and 2008 about 7.2 MT (46%) of edible oil had to be imported (Jain & Sharma, 2010). From a socioeconomic point of view, it is therefore more reasonable to divert biofuel production to plants, which produce high amounts of non-edible oil and may be cultivated on marginal lands. Out of a list of oil-seed plants with such properties, *Jatropha curcas* is one of the most promising ones (Table 1.2.1, Singh & Singh, 2010).

Due to its hardiness *Jatropha curcas* supposedly possesses other beneficial characteristics currently under research:

1. *Jatropha* is thought to have potential to reclaim land that has before been eroded and is of no use to the local population. Therefore, the plant has the potential to pro-

vide employment, improve the environment and enhance the quality of rural life (Makkar & Becker, 2009).

Table 1.2.1 Different oil-seed species producing unedible oil for biofuel in India.

Species	Oil fraction %	Seed estimate (10^6 tons / yr)	Oil yield (tons / ha / yr)
Jatropha	50-60	0.20	2.0 - 3.0
Castor	45-50	0.25	0.5 - 1.0
Mahua	35-40	0.20	1.0 - 4.0
Sal	10-12	0.20	1.0 - 2.0

Data taken from Singh & Singh, 2010

2. When degraded lands are reclaimed, *Jatropha* may contribute to carbon sequestration. Over the period of four years, 2500 kg C / ha was redeposited in the soil by *Jatropha* in a study conducted in Andhra Pradesh, India, significantly improving soil microbial activity and respiration as well as water retention capacity compared to a control soil (Wani et al., 2012).

3. Model projections further show that a 10.000 km^2 plantation of *Jatropha* set up on dry coastal area land could further lead to a decrease in local temperature accompanied by a continuous onset or increase in local rainfall. In an optimal scenario, this could lead to permanent woodland or forest without artificial irrigation (Becker et al., 2013).

Nonetheless, there are also critical voices debating whether *Jatropha curcas* is a truly sustainable source of biofuel. Critics argue that the plant may have the ability to grow in low precipitation areas, but when it does, only produces very small amounts of oil (Openshaw, 2000; Axelsson et al., 2011). In fact, *Jatropha* is thought to use up 5 times more water than corn to reach the same amount of oil production. For 1 liter of biodiesel from *Jatropha*, around 20,000 liters of water are necessary. Rapeseed, grown in areas with high precipitation, only needs 14,000 liters.

Whether or not *Jatropha* is a viable crop for biofuel production remains to be clarified. Programs are currently running to optimize oil yield in *Jatropha* in order to even out its water foot print (Kesava Rao et al., 2012).

Due to the fact that *Jatropha* is inedible, plantations in the immediate vicinity of large agglomerations, such as Luxor, Egypt, may offer another potential application. There, a 100 ha test plantation was irrigated with sewage water from the city (Becker et al., 2013). In Islamic countries, sewage water is prohibited for the production of food crops, hence *Jatropha* may provide an opportunity for its sustainable use.

The processing steps of *Jatropha curcas* seeds are laid out in Figure 1.3.1. The seed of *Jatropha curcas* is mechanically pressed to gain the first batch of oil. This way, about 260 kg oil per ton of seed is gained. Subsequently, the remaining press cake can be treated with solvent to gain about another 90 kg. The total average crop is therefore around 350 kg of oil / ton of seed and 650 kg of press cake / ton of seed (Makkar & Becker, 2009). It is this 65% of seed cake that was evaluated within this thesis.

In 2008, global *Jatropha curcas* plantations covered 900 000 ha with a seed yield between 0.5 and 12 tons / ha and an average of 2 tons / ha translating to a total of 1.8 MT (Borman et al., 2013). Reported data on seed yield vary (cf. Table 1.2.1, Figure 1.3.1), but assuming an oil yield of 35%, global production would yield 1.17 MT of press cake or *Jatropha curcas* kernel meal potentially available as a feed ingredient.

1.3 Characteristics of *Jatropha curcas* kernel meal (JKM)

1.3.1 Crude protein and essential amino acid content

The crude protein content of JKM is considerably higher than in soy bean meal and can be compared to the protein content of fish meal or soy protein concentrate (Table 1.3.1). The essential amino acid (EAA) composition in JKM can be consid-

ered valuable as it has a similar profile to fish meal. JKM contains more methionine than soy bean meal. However, it has less lysine and only around half of the lysine provided by fish meal. Lysine and methionine are widely considered the first limiting amino acids when using plant-based protein sources as these are not found in high concentrations in most plants (Gatlin et al., 2007).

There are significant differences in nutrient composition of different batches of JKM,

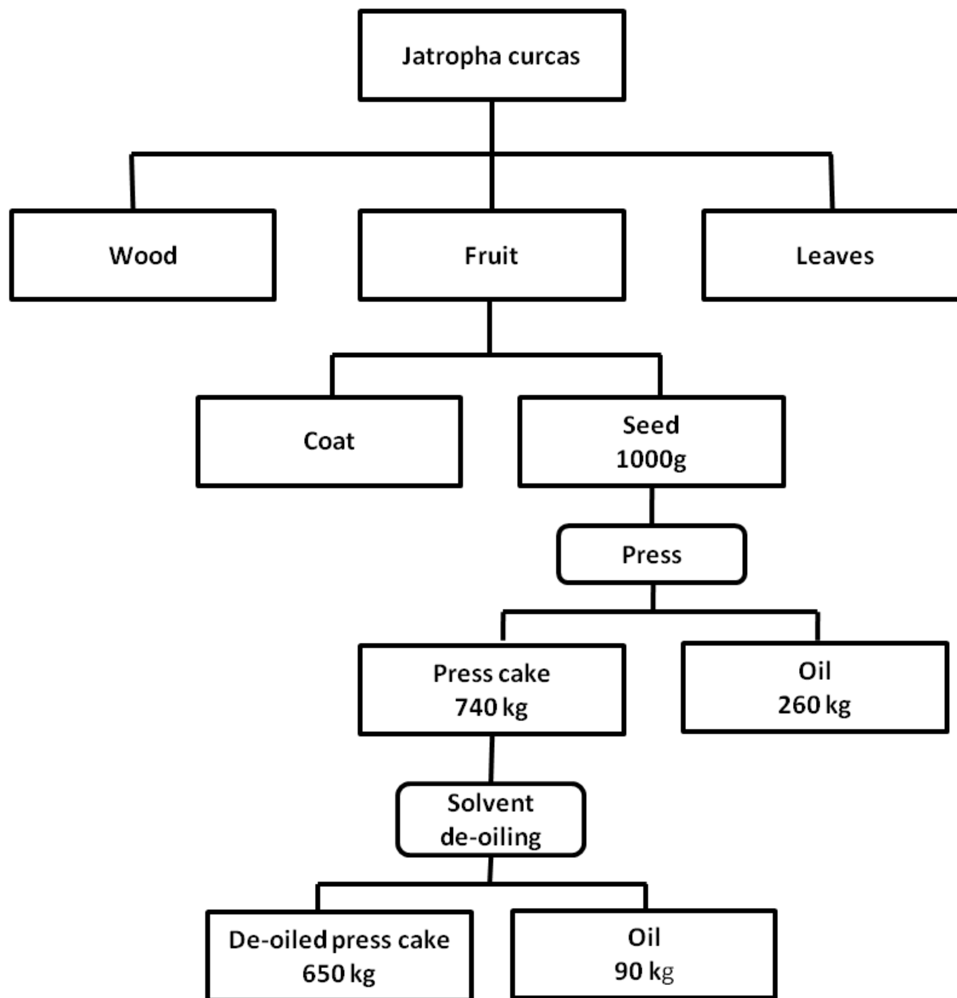


Figure 1.3.1 Flow chart of processing of *Jatropha curcas* seeds with relative yields of oil and de-oiled press cake.
Values from Makkar & Becker, 2009

dependent on the exact method of detoxification and the amount of kernel husk present in the meal. Crude protein content may vary between 50.9% and 66.5% (Table 1.3.1 and Table 1.3.2). The difference in essential and non-essential amino acid composition of two batches is shown in Table 1.3.2. The two most important essential amino acids, lysine and methionine, are present in higher concentrations in batch 1 (3.42 g / 100 g protein and 1.99 g / 100 g protein, respectively) than in batch 2 (3.16 g / 100 g and 1.45 g / 100 g, respectively). Both these amino acids are however present in lower fractions in JKM than required by carp or tilapia, which implies that they need to be supplemented or added through other ingredients to achieve optimal growth. It is of essential importance to know the exact EAA composition of a batch of JKM when formulating a diet with optimal EAA requirements for the fish.

1.3.2 Fat and ash content

Jatropha curcas kernel meal is generally defatted as the valuable kernel oil is nearly completely used for biodiesel production. As seen in Figure 1.3.1, the amount of fat remaining in the meal depends on the extraction methods applied. Only a mechanical extraction would leave about 12% of lipids in the meal. An additional solvent extraction step lowers this amount to almost 0%. Complete extraction of fat is essential in order to prevent saponification when treating the meal with NaOH for detoxification (see 1.3.4). The trials presented in this work were all conducted with JKM containing negligible, residual lipid of about 0.2%.
JKM ash content of 13.7% is higher than in other plant-based feedstuffs like, for example, soybean meal (5.7%, Table 1.3.1). Reason for this are the higher total phosphorus and calcium contents found in JKM. Calcium and phosphorus are essential minerals for bone mineralization and therefore supplemented to most commercial diets. The high phosphorus content of JKM, if made available to the fish, can represent an additional benefit of JKM compared to other plant-based feedstuffs.

However, if not made available to the fish and excreted (see Chapter 1.3.3), this additional phosphorus may lead to eutrophication of the environment and act as a limiting factor in dietary JKM inclusion. A detailed mineral composition of JKM compared to other feedstuffs can be found in Table 1.3.3.

Table 1.3.1 Comparison of nutrients and essential amino acids of different plant-based feedstuffs to fishmeal

Nutrient	**Fishmeal**	**Soybean meal**	**Soy protein concentrate**	**Soy protein isolate**	**Wheat gluten**	**Rapeseed protein concentrate**	***Jatropha curcas* kernel meal**
Dry matter (g / kg)	928	894	917	915	950	942	945
In meal, (g / kg)							
CP	748	474	679	885	810	710	665
Fat	122	11	1	2	56	22	11
Ash	119	57	63	39	10	92	137
Gross energy, MJ / kg	21	17		21	22	25	18
Amino acids g / 100 g protein							
Arginine	6.7	7.4	7.5	6.8	3.6	10.5	10.5
Histidine	1.9	3.0	2.4	1.9	1.9	4.2	3.3
Isoleucine	4.0	4.2	4.6	4.7	3.5	6.0	4.0
Leucine	7.3	8.4	7.9	8.5	7.0	11.0	7.0
Lysine	7.2	6.5	6.1	6.1	1.5	8.0	3.5
Met + Cys	3.4	4.2	2.9	2.1	3.8	2.9	1.6
Phe + Tyr	6.5	9.3	8.9	8.9	8.2	6.0	4.6
Threonine	4.4	4.2	3.9	4.0	3.7	6.3	3.3
Tryptophan	1.0	1.4	1.5	1.2	0.9	2.0	1.1
Valine	5.0	4.6	5.1	4.6	3.9	7.6	4.8
Source	Storebakken et al., 2000	Riche & Williams, 2011	Refstie et al., 1999	Riche & Williams, 2011	Storebakken et al., 2000	Slawski et al., 2011	Kumar et al., 2010

Table 1.3.2 Comparison of two essential and non-essential amino acid compositions of two different batches of JKM as well as essential amino acid requirements of carp and tilapia

EAA	**Batch 1[1)]**		**Batch 2[2)]**		**Carp[3)]**	**Tilapia[3)]**
	%	**g / 100g CP**	**%**	**g / 100g CP**	**g / 100g CP**	**g / 100g CP**
Arginine	6.27	10.5	5.26	10.5	5.4	3.8
Histidine	2.08	3.46	1.25	2.49	1.6	3.2
Isoleucine	2.42	4.05	2.27	4.51	3.2	3.2
Leucine	4.35	7.27	3.42	6.80	4.5	6.1
Lysine	2.05	3.42	1.59	3.16	7.0	5.1
Phenylalanine	2.76	4.61	2.23	4.43	4.2	3.5
Methionine	1.20	1.99	0.73	1.45	2.2	2.2
Threonine	2.23	3.72	1.74	3.46	4.8	3.5
Tryptophan	0.68	1.14	0.54	1.07	1.0	1.0
Valine	2.77	4.62	2.53	5.03	4.5	4.8
NEAA						
Aspartic acid	5.76	9.61	4.49	8.93		
Serine	3.01	5.03	2.27	4.51		
Glutamic acid	9.52	15.9	7.64	15.2		
Proline	2.63	4.39	2.07	4.12		
Glycine	2.78	4.63	2.14	4.25		
Alanine	2.97	4.96	2.3	4.57		
Cysteine	1.71	2.85	0.7	1.39		
Tyrosine	1.60	2.67	1.2	2.39		
Total CP (% / DM)	**59.9**		**50.3**			

[1)] Analyzed by Prof. Rodehutscord, Institute for Animal Nutrition, University of Hohenheim, Germany, Date of Analysis: 1-11-2011, unpublished

[2)] Analyzed by LUFA, Kiel, AgroLab Group, Germany. Date of Analysis: 11-11-2013, unpublished

[3)] Requirement data from NRC (2011) recalculated based on 32% dietary protein content

Table 1.3.3 Total mineral-content of JKM compared to fishmeal, soybean meal and rice bran

Mineral	Unit	JKM[1)]	Fishmeal[2)]	Soybean meal[3)]	Rice bran[4)]
P	%	1.43	2.88	0.69	0.66
Ca	%	0.80	5.19	0.34	0.25
Mg	%	0.93	0.15	0.30	0.20
Na	%	0.67	0.41	0.02	0.69
K	%	0.64	0.70	2.14	0.28
Mn	mg/kg	79.4	37.0	36.0	12.5
Fe	mg/kg	2024	544	176	74
Cu	mg/kg	42	10.3	20	6.4
Zn	mg/kg	84	144	55	68

Values in % DM

[1)] Analyzed by ICP-MS, Institute of Aquaculture, Stirling, UK, unpublished

[2)] Menhaden, mechanically extracted (NRC, 2011)

[3)] Solvent extracted without hulls, 48% CP (NRC, 2011)

[4)] NRC, 2011

1.3.3 Anti-nutritional factors (ANFs)

Anti-nutritional factors have been defined as “substances, which by themselves, or through their metabolic products arising in living systems, interfere with food utilization and affect the health and production of animals” (Makkar, 1993). JKM has the potential to become a valuable feed ingredient in livestock and fish feed in the future, however, it also contains several anti-nutritional factors, which could potentially lead to lower nutrient availability. Table 1.3.4 provides an overview of anti-nutritional factors present in *Jatropha curcas*.

Anti-nutritional factors cannot be regarded from an isolated viewpoint. They may interact with one another and some studies have even noted beneficial effects of a certain concentration of individual anti-nutritional factors, such as trypsin inhibitor in channel catfish (Wilson and Poe, 1985). The following is a description of the most important anti-nutritional factors present in *Jatropha curcas.*

Table 1.3.4 Anti-nutritional factors in toxic type of *Jatropha curcas* kernel meal (untreated).

Component	Content
Phorbol esters (mg/g kernel)[1]	2.79
Total phenols (% tannic acid equivalent)[1]	0.36
Tannins (% of tannic acid equivalent)[1]	0.04
Phytate (% of dry matter)[1*]	9.4
Saponins (% diosgenin equivalent)[1]	2.6
Trypsin inhibitor (mg trypsin inhibited/g sample)[1]	21.31
Lectin activity (1/mg of meal that produced haemagglutination/ml assay medium)[1]	51-102
Oxalate (%)[2)]	2.44

[1)] Data from Makkar & Becker, 2009

[2)] Data determined by HPIC, Prof. Lohaus, Wuppertal, Germany, unpublished

* Phytate analysis in the present work proved this value to be incorrect. True phytate content is 2.58% (see Chapter 4.3)

Tannins

Tannins are present at very low concentrations in JKM. They are a group of molecules with a polyphenolic structure, which are supposedly produced by plants as a feeding protection mechanism. Tannins have the ability to bind amino acids and make them precipitate. The molecule plays an important role in the ripening of fruit or the aging of wine (Francis et al., 2001).

There is a difference between condensed tannins and hydrolysable tannins, the latter one being more dangerous to fish as their breakdown products enter the blood stream and may lead to organ deterioration. In carp, condensed tannins are tolerated at least until levels of 2% (Becker and Makkar, 1999). It is therefore unlikely that the concentration of 0.04% in JKM has any impact on fish health or growth.

Saponins

Saponins are a group of molecules with particular abundance in various plant species where they serve as anti-feeding agent against plant-eating animals. They have the structure of a glycoside with a hydrophilic sugar on one side and a lipophilic triterpene on the other side. This is also the reason why they have a soap-like foaming effect, when shaken in water. Their name is derived from the soapwort plant

(Genus *Saponaria*), owing its name to the fact that its roots have historically been used as soap. When added to water, the toxicity for fish is caused by the detergent action at the gill epithelium (Francis et al., 2001). In many countries tea seed cake is used in aquaculture ponds to kill remaining fish before stocking new fingerlings, the active ingredient being saponins contained in tea seed cake at 7-8%. 100 ppm of tea seed cake resulted in 100% mortality of tilapia within 5-6 h (De et al., 1987).

There have been studies on saponins in cultured fish diets with contradicting results. While some studies show damages on the digestive epithelia of salmon (Bureau et al., 1998) and slower growth in tilapia (Olvera Novoa et al., 1990), which are thought to derive from saponin, other studies suggest that low levels of saponin do not have any adverse effects on growth or might even be growth-promoting as the detergent characteristics reduces feed viscosity and may thus support digestion (Francis et al., 2001; Stadtlander, 2012).

Trypsin Inhibitor (TI)

Protease inhibitors are molecules, which prevent the function of proteases, such as trypsin, in the digestive tract. With around 21.31 mg / g (Table 1.3.4) JKM has a relatively high content of trypsin inhibitor, compared to, for example, soy with 5-8 mg / g (Hart et al., 2010). In various experiments the impact of trypsin inhibitor on amino acid availability has been studied with very different results. Some trials conclude that trypsin inhibitor does not influence the assimilation of amino acids, while other studies show a definite reduction of growth proportional to trypsin inhibitor concentration in the diet.

When detoxified, JKM still contains between 1.3 to 8.3 mg TI / g. In a trial on carp, this did not have any influence on growth performance compared to non-detoxified JKM implying that carp can sustain high levels of TI in the diet (Makkar and Becker, 1999). On the contrary, Wee and Shu (1989) showed that for tilapia concentrations of 1.6 mg TI / g diet already retarded growth. Similar findings were observed for

rainbow trout, which proved to be highly sensitive to TI isolated from soy bean meal (Sandholm et al., 1976).

It has been suggested that fish can upregulate their trypsin production in reaction of higher trypsin inhibitor concentrations (Olli et al., 1994) and it seems like the benchmark at where concentrations become critical lies around 5 mg / g diet for most species (Francis et al., 2001).

Lectin

Lectins are proteins that have the ability to bind specifically to certain sugar moieties. They are found in many different legume seeds and -despite being proteins- demonstrate a level of resistance to enzymatic breakdown in the digestive tract (Grant et al., 1991).

The level of lectin activity is similar between the toxic and the non-toxic type of *Jatropha*, suggesting that it is not one of the main toxic components in the plant. High concentrations of lectin (e. g. 51 haemagglutination units) had only minor effects on the growth of carp when compared to diets with low concentrations (e. g. 1.2 haemagglutination units, Makkar & Becker, 1999). Lectins may have a morphological impact on the digestive system of fish as indicated for Atlantic salmon fed full-fat soy bean meal containing high levels of lectins (van der Ingh et al., 1991). Grant et al. (1991) pointed out several deterring effects to the digestive epithelia when soy bean meal containing soybean lectins were fed to other animals.

For JKM, Aregheore et al., (1998) managed to reduce the lectin concentration by moist heat treatment of 100°C for 10 minutes from 102 to 1.17 haemagglutination units.

Non-starch polysaccharides (NSPs)

The term “non-starch polysaccharides” encompass a wide range of polysaccharide molecules, excluding starch (alpha-glucan). After Bailey (1973), they are classified in three groups, celluloses, non-cellulosic polymers and pectic polysaccharides. The

anti-nutritional effect of NSPs is assumed to be in a multitude of factors related to physiological changes of the digestion process and morphological changes in the gut. The length and weights of digestive organs increase in African catfish (*Clarias gariepinus*) when fed diets containing NSPs from guar gum (Leenhouwers et al., 2006). The viscosity of the digestive areas seems to be dependent on the type and quantity of NSPs. NSPs may bind with the intestinal brush border and increase the thickness of the unstirred water layer thereby decreasing digestive efficiency (De Lange, 2000). In trials for Atlantic salmon, inclusion of soybean NSPs to the diet led to higher viscosity and lower amino acid and fatty acid digestibility (Refstie et al., 1999). Increased viscosity also increases the retention time of feed in the gut, which can lead to fermentation processes and changes in the micro flora (Choct et al., 1996). So-called volatile fatty acids (VFA) are produced, which alter the pH of the intestinal tract and may lead to adverse effects in the micro flora in the long term. Furthermore, high NSP concentrations in diets lead to a shortening of villi length, which lead to reduced surface area for digestive processes and consequently lower nutrient uptake (Hopwood et al., 2002).

JKM has a total NSP content of 160 g / kg dry matter, out of which 65-76 g / kg is cellulose. Compared to soybean meal, this is slightly less (total NSP: 217 g / kg; cellulose: 62 g / kg). Still, NSPs in JKM may be responsible at least partly for some adverse effects in fish digestion (Sinha et al., 2011).

Phytate

Phytate is a common anti-nutritional factor in plant-based feedstuffs (Figure 1.3.2). JKM has a phytate content between 7 – 9%, which is more than 5 times as much as soy (Kumar et al., 2011b). Phytate is also known as phytic acid or myo-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate (IP6). It is the principal storage form of phosphorus in plant seeds. In oilseed meals between 34% and 66% of total phosphorus may exist in form of phytate (Eeckhout & de Paepe, 1994). There is thought to be a connection

between the ability of *Jatropha* to thrive in low-nutrient soils and its high phytate content. Aside from phosphorus, it is also thought to store cations, such as calcium, magnesium, iron and zinc. In acidic environments, such as the tilapia stomach, half of the phosphate moieties of phytate are negatively charged and may therefore chelate with the respective cation (Riche et al., 2004). This solid bond leads to problems when substances with high phytate content are digested by humans or animals. In particular, seeds are not suited as a source for minerals despite their high mineral contents, because of high phytate concentrations. Minerals in general are taken up in the small intestine and phytate can impede the uptake in these regions (NRC, 2011). Additionally, some minerals such as calcium and magnesium are excreted by the pancreas as of a recycling process and are taken up again in the intestine. As phytate may irreversibly bind to these minerals, a diet based on feed ingredients with high phytate content, such as soy, may cause serious mineral deficiencies. When replacing fish meal with plant based diets, it is therefore necessary to pay close attention to their phytate content (Kumar et al., 2012b).

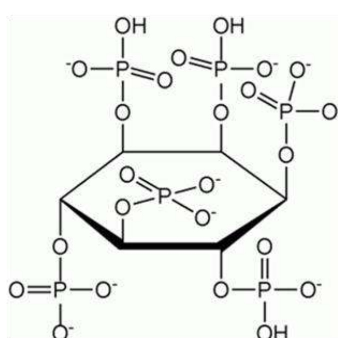


Figure 1.3.2 The molecular structure of phytate.

However, phytate is not only considered to cause deficiencies in mineral uptake, there are also reports that phytate forms complexes with amino acids rendering them indigestible by proteolytic enzymes. Spinelli et al. (1983) fed a diet containing 5 g / kg synthetic phytic acid to rainbow trout and observed the formation of phytate-

protein complexes resulting in slower growth rates. In the same study, *in vitro* tests showed that there was only partial hydrolyzation of phytate/protein (casein) complexes by pepsin. *In vivo* tests on rainbow trout where casein was replaced by a casein phytate complex showed 6.6% reduction in diet digestibility. The phosphorus content in the diets applied in this study was between 0.9 and 1.1%.

Phytase (myo-inositol hexakisphosphate phosphohydrolase) is an enzyme, which catalyzes the hydrolysis of phytate to pentakisphosphate (IP5) or to less phosphorylated myo-inositol phosphates. The complete hydrolysis produces one molecule of inositol and six molecules of inorganic phosphate (Rao et al., 2009). Phytases are classified in three different types: 3-phytase, 5-phytase and 6-phytase dependent on the location of the binding site of the phytase at the phytate molecule. 3-phytase dephosphorylates phytate at the third phosphate group, 5-phytase at the fifth and 6-phytase at the sixth group. Different organisms produce different phytase types. 3-phytases, for example, are produced by *Aspergillus niger*, *Neurospore crassa* or *Pseudomonas* species. 5-phytases are produced by *Medicago sativa* or *Phaseolus vulgaris*, whereas 6-phytases are produced by *E. coli* or *Paramecium* (Rao et al., 2009*)*.

There are several producers of commercial phytase. Commercial phytase is supposed to lower the phytate concentration in diets for livestock, poultry and fish to improve bioavailability of nutrients. The two most widely used commercial phytases are Ronozyme by DSM and Natuphos by BASF. While adding phosphorus to diets can have the same effect, it is more ecological to break up the phytate bond and use the disassociated phosphorus. In an experiment by Weerd et al. (1999) it was discovered that addition of phytase to soy-bean based catfish diets helped minimize the phytate-caused deficiencies of phosphorus and therefore improved growth rate and feed utilization. Fox and Davies (2011) showed that phytase isolated from corn can help rainbow trout juveniles to strengthen their scale and vertebral morphology.

The phytase Ronozyme added to plant-based diets with no additional phosphorus resulted in better growth rate and phosphorus utilization in tilapia (Liebert and Portz, 2005). Cao et al. (2008) found that best growth rates were achieved when 12.5 g / kg mono calcium phosphate was added in conjunction with 1000 U / kg phytase to a diet based on de-oiled soybean meal. Growth in this trial was just as fast as the in-organic phosphorus control diet and clearly faster than diets without phytase sup-plementation. Phosphorus retention and apparent digestibility was also significantly better in diets containing phytase.

While the effect of phytase on mineral availability is well-accepted, there are also references that phytase may have an effect on protein digestibility in some fish spe-cies. Vielma et al. (2004) found that adding phytase to soybean meal based diets improved protein digestibility by 3.2%-units, but not lysine digestibility. Debnath et al. (2004) showed that protein digestibility at a phytase activity of 500 U / kg was best in a soybean based diet for *Pangasius* fingerlings. In their study, protein digestibility was directly proportional to the amount of phytase added up to the point where 500 U / kg were reached. The protein digestibility was about 20%-units better than the non-treated diet.

Phytase can be applied to feed in different ways. One way is to incorporate phytase directly in the diet. In commercial feed production, however, using an extruder, this method is not recommendable as phytase is not heat stable and may disintegrate under the high temperatures of the extrusion process. Cheng and Hardy (2002) ob-served that mineral availability in combination with phytase decreased when extru-sion processing methods were applied in diets made of barley, corn-gluten meal or wheat. Alternatively, phytase can be applied to the plant ingredient before the extru-sion process. Nwanna et al. (2008) managed to reduce phytate content in soybean and wheat gluten based diets from 0.48% down to 0.015% by pre-incubating the plant feedstuffs at 40°C for 15.5 hours. They monitored significantly better growth of

carp in incubated diets than in non-incubated ones. However, pre-incubating of plant feedstuffs is an extra processing step that is likely to be too costly for commercial production. Another common method is to spray phytase on the diet after pelleting. This way, the high temperatures are circumvented. In a direct comparison, Wang et al. (2008) demonstrated for rainbow trout that pre-incubating soybean-based diets significantly lowered phytate content, while spraying the enzyme only had a small effect on the phytate level. In this experiment, spraying the diet however, showed an improvement in feed conversion ratio and specific growth rate, while the pre-treatment diets did not. Vielma et al. (2004) also sprayed phytase on the diet of rainbow trout and also demonstrated improved growth. Another method is to mix phytase with water and alginate and drip this mixture in a chitosan/calcium chloride solution to form microcapsules. These microcapsules protect the phytase and therefore it withstands higher temperatures than usual. However, it has been shown that this technique results in lower abilities of phytase to liberate phosphorus and to increase protein digestibility than the not encapsulated form, presumably because the microcapsules prevent the phytase from reaching its point of action (Vandenberg et al., 2011).

Oxalate

The present thesis revealed high concentrations of oxalate in JKM through HPIC analysis of the acid extract (Chapter 4.3). Oxalate (Figure 1.3.3) is the salt of oxalic acid, produced and stored by several crop plants to regulate intracellular calcium concentration, as a plant defense mechanism and as a heavy metal detoxification agent (Francesci et al., 2005). The production of oxalic acid in the plant is mainly through the oxidation of glyoxylate and glycolate as a by-product of the glyoxylate cycle (Francesci et al., 2005). Dependent on plant species, oxalate accumulates as soluble oxalate bound to monovalent counter ions, such as sodium, potassium or ammonium or as insoluble oxalate formed with divalent cations, such as calcium,

magnesium and iron or as a combination of the two (Libert & Franceschi, 1987; Savage et al., 2000).

In humans and animals, oxalate is also produced through the metabolism of ascorbate and glycine (Noonan & Savage, 1999). At least in humans, there is no enzyme to further break down oxalate, so it has to be disposed of through the kidney. It has been shown that only 10-15% of urinarily excreted oxalate is derived from the diet, while the rest comes from internal production of oxalate (Noonan & Savage, 1999). The anti-nutritional properties of oxalate have not yet been discussed in connection to aquaculture feeds, though some authors have speculated about potential anti-nutritional effects (Yousif et al., 1994; Francis et al., 2001). Oxalate contents of several feed ingredients are shown in Table 1.3.5.

Table 1.3.5 Soluble oxalate contents of selected fodder crops (% DM).

mmon Name	Scientific Name	Soluble oxalate
Saltbush[a]	*Atriplex halimus*	1.0 - 3.0
Rice[a]	*Oryra satiua*	1.0 - 2.5
Alfalfa[a]	*Medicago sativa*	0.96 - 1.10
Soybean meal[b]	*Glycine max*	0.20
DJKM[1,b]	*Jatropha curcas*	2.44

[1]Detoxified *Jatropha curcas* kernel meal

[a] Value taken from Libert & Francechi, 1987

[b] Value determined by HPLC

While monovalent cation-oxalate molecules are soluble in gastric pH, divalent cation-oxalate bonds are not and this may lead to shortages in the assimilation of these types of minerals in the digestive tract when fed with a high-oxalate diet (Rahman et al., 2013). This has been shown for sheep, which suffered lower bioavailability of calcium when fed high-oxalate containing diets (Rahman et al., 2011). There are also immediate, toxic effects of oxalate. When oxalate is present in a diet to a high

extent along with a low concentration of divalent minerals, then oxalate stays in solution and is taken up in the blood stream. Here, it may accumulate in the kidney where it then binds to divalent cations, especially calcium, and precipitates. This leads to kidney stones and potentially kidney failure. Long-term uptake of high oxalate concentrations that are not immediately toxic may still lead to slow formation of stones and cause kidney disfunction as well as may lead to mobilization of bone material to compensate for shortages in minerals (Rahman et al., 2013). Therefore, it has been recommended that for ruminants dietary oxalate consumption should be less than 2% in order to avoid oxalate poisoning and less than 0.5% for non-ruminants (Rahman et al. (2013). Anti-nutritional effects of dietary oxalate for freshwater fish may include lower calcium or magnesium availability and/or the formation of kidney stones. Furthermore, oxalate binding to calcium has been shown to be influenced by amino acids, so it may be speculated that there could also be an interaction of oxalate and amino acids in the digestive tract (Sargut et al., 2010).

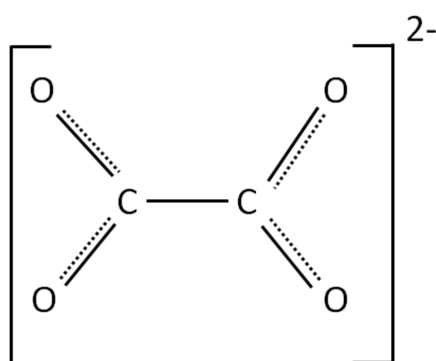


Figure 1.3.3 The molecular structure of oxalate.

Phorbol esters

Phorbol esters are the main toxic substance in *Jatropha* oil and meal. Dependent on extraction method and origin of the meal, concentration in oil varies between 2.1 g / kg and 2.8 g / kg (Pradhan et al., 2011). As shown in Table 1.3.4, the concentration of phorbol esters in the press cake is comparable.

The toxicity of phorbol esters lies in the imitation of diacyl glycerol (DAG), an activator of protein kinase C, which regulates cellular signal transduction pathways and other metabolic activities, such as cell growth (Goel et al., 2007). In a normally functioning system, DAG is rapidly hydrolyzed after activating protein kinase C. Phorbol esters, however, cannot be metabolized by the cell. It is therefore assumed that phorbol esters over-activate protein kinase C and convert it into a constitutive form that is irreversibly inserted into the membrane. This can lead to uncontrolled cell proliferation and amplify the efficacy of carcinogenic components (Mosior & Newton, 1995).

1.3.4 Detoxification of *Jatropha curcas* press cake

Unlike other toxic or anti-nutritional substances, such as trypsin inhibitor or lectins, which can be destroyed through dry or moist heating, phorbol esters are heat-stable and therefore need to be destroyed via a different method. The detoxification process as patented by Makkar & Becker (2011) comprises the following steps:

1. A 90% methanol solution containing 0.1 M NaOH is added to JKM at a ratio 1:10 (w / v).
2. The mixture is heated to 65-70° for 30 minutes.
3. The mixture is filtered and the filtrate is discarded.
4. The residue is washed with 90% methanol (added at 1.2 times the weight of the JKM originally used).
5. 90% methanol is added at 1:10 (w / v), heated again for 30 minutes, filtered and washed with 90% methanol.
6. The washed residue is dried to 75% moisture and autoclaved at 121°C for 15 minutes.
7. The product is dried to obtain final detoxified JKM.

After the detoxification steps, phorbol esters cannot be detected by HPLC with detection limit of 3 ppm. While this detoxification method is the method of choice for JKM used in the present thesis and is effective in erasing phorbol esters from the meal, it also consumes large amounts of NaOH and methanol and may therefore turn out to be uneconomical when scaled up. Further, it can be subject to modification as shown by Guedes et al. (2014) who removed phorbol esters by 97.3% using a methanol/ethanol mixture. Other detoxification methods are currently under investigation. Phengnuam & Suntornsuk (2013) investigated bacterial fermentation as a potential detoxification method and found submerged fermentation for 5 days with *Bacillus licheniformis* to reduce phorbol esters by 62%. Solid-state fermentation with *Apergillus versicolor* CJS-98 reduced phorbol esters from 0.083% to 0.015% (82% reduction, Veeradhadrappa et al., 2014). Wang et al. (2013) isolated a novel bacteria strain and applied a mathematical model in combination with a feeding trial of carp fingerlings to determine the reduction of general toxicity (not just phorbol esters) of JKM after fermentation and concluded that it was reduced by 97%. Another method using hydroxyl radicals (·OH) produced by plasma irradiation completely eradicated phorbol esters from JKM in aqueous solution (Kongmany et al., 2013).

1.4 Previous work on JKM in fish diets

JKM is a novel feed ingredient that has undergone only very limited research. Unlike other oilseed cakes, such as from soybean or rapeseed, JKM is not used by commercial feed producers as of yet.

In a recent study by Workagegn et al. (2013) JKM was applied in diets without any treatment or with only a heat treatment step (120°C, 45% moisture, 15 minutes) before being mixed at various percentages to diets for Nile tilapia. Without any treatment, tilapia showed significantly higher mortality and slower growth rate already at a 10% inclusion level of JKM in the diet. For heat-treated JKM, 10% inclusion level

produced no differences in mortality and only insignificantly slower growth, but inclusion levels of 20% resulted in higher mortality and significantly slower growth. These results show the necessity of detoxification of JKM before application to a diet.

Detoxified JKM has been tested as a fish feed ingredient in more detail only recently. Unheated and heated JKM of non-toxic origin (no phorbol esters) was tested by Makkar & Becker (1999) in a diet for carp. In this study, heated JKM had significantly reduced trypsin inhibitor and lectin concentrations, but body weight gain, protein efficiency ratio and protein productive value showed no difference between heated and unheated meal. This shows that trypsin inhibitor and lectins do not necessarily reduce the nutritional value of JKM in diets for carp.

After complete detoxification procedures were established, a variety of experiments were conducted with detoxified JKM. Kumar et al. (2010) replaced 75% of fishmeal in carp diets with detoxified JKM and found that body mass gain was equal to the fish meal control and significantly better than a diet with 75% soybean meal. In a different experiment by the same authors (Kumar et al., 2011b), carp were fed diets with JKM exposed to two different durations of detoxification processes, one for 30 minutes, the other one for 60 minutes. JKM replaced fish meal at 50% and 75% respectively for both detoxification durations. Unlike in the first experiment, this experiment showed equal growth between control diet and JKM-based diet only in the 60 minutes detoxified, 50% replacement diet, while all other diets showed reduced growth. These inconsistent results demonstrate the need for more research in the area.

JKM experiments have also been conducted for other species. For tilapia, 62.5% fish meal replacement showed equal growth rates compared to control diets (Akinleye et al., 2011; Kumar et al., 2012a). For rainbow trout (*Oncorhynchus mykiss*), on the other hand, replacing fish meal at 62.5% resulted in lower growth rates than 50% addition (Kumar et al., 2011c).

In the experiments conducted so far, test fish cannot convert more than 75% JKM towards similar growth rates as fish meal. In all of these experiments, the question remains why, despite elimination of phorbol esters, lectins and trypsin inhibitors, JKM is still less nutritious than fish meal. One key role here is attributed to the anti-nutritional factor phytate. In a study by Kumar et al. (2011a) a phytate-rich fraction was isolated from JKM and added to a casein-based standard diet for tilapia, so that phytate concentration reached 1.5% and 3% respectively. Two treatments also containing 1.5% and 3% of phytate but additionally 1500 (U / kg) phytase were included. Results showed that growth was significantly impaired when the phytate rich fraction only was added, but this could be compensated by adding phytase to the diet. It is therefore assumed that phytate plays a major role in impeding the digestion process.

1.5 Aims and scopes of the project

The economical success of commercial *Jatropha curcas* cultivation be it for local subsistence farmers or multi-national conglomerates, is closely connected to the value of JKM. With its high phosphate and nitrogen content, JKM undoubtedly makes a good fertilizer and this is what it is used for today in most sites (Achten et al., 2008). However, its value would increase manifold if it could be included in commercial diets for aquaculture or livestock. Domestication of *Jatropha curcas* is at its infancy with the consequence of high varieties in crop and therefore oil yields (Achten et al., 2008). This makes it difficult for farmers or investors to determine the profitability of a commercial venture. The enhanced value of JKM by enabling its use as a feedstuff could considerably influence commercialization of *Jatropha curcas* as a crop.

Research on using JKM in aquaculture diets has been conducted on a limited basis. As stated above only very few work groups have ever dealt with the problem, mainly

due to the fact that detoxification of the seeds has only recently been conducted successfully. The works presented in 1.4 are a first step, but cannot be regarded as complete. The reason for choosing carp and tilapia as the selected fish species in the present work is that these species are the most cultured ones in countries potentially suitable for *Jatropha curcas* cultivation (FAO, 2012).

It is the aim of the present study to complement previous findings by dealing with the following topics:

1. Replacement level of fishmeal by JKM in diets for carp and tilapia

The present work was initiated to determine the optimal replacement levels of fishmeal with JKM for carp and tilapia.

2. JKM as a protein source in practical diets for carp and tilapia

Dependent on life stage, type of culture and country, commercial diets for carp and tilapia already contain very low amounts of fish meal with the predominant share of protein coming from other plant-based sources (Tacon & Metian, 2008). Another aim of this thesis was to investigate whether inclusion of JKM could further lower fishmeal content in practical diets for carp and tilapia.

3. The role of phytate and phytase in JKM based diets

The third aim was to determine and identify the effects of the anti-nutritional phytate component of JKM on fish development and whether application of commercial phytase can prevent / improve these effects.

4. Dietary oxalate as an anti-nutritional factor in carp and tilapia

Analysis of anti-nutritional components revealed comparatively high amounts of oxalate in JKM (see Table 1.3.4). The present thesis also determines the influence of oxalate as a potential anti-nutritional factor on carp and tilapia development.

1.6 References

Achten, W. M. J., Verchot, L., Franken, Y. J., Mathijs, E., Singh, V. P., Aerts, R., & Muys, B. (2008). *Jatropha* bio-diesel production and use. *Biomass and Bioenergy*, *32*(12), 1063–1084.

Akinleye, A. O., Kumar, V., Makkar, H. P. S., Angulo-Escalante, M. A., & Becker, K. (2011). *Jatropha platyphylla* kernel meal as feed ingredient for Nile tilapia (*Oreochromis niloticus* L.): growth, nutrient utilization and blood parameters. *Journal of Animal Physiology and Animal Nutrition*, *96*(1), 1–11.

Aregheore, E. M., Makkar, H. P. S., & Becker, K. (1998). Assessment of lectin activity in a toxic and a non-toxic variety of *Jatropha curcas* using latex agglutination and haemagglutination methods and inactivation of lectin by heat treatments. *Journal of the Science of Food and Agriculture*, *77*, 349–352.

Axelsson, L., Franzén, M., Ostwald, M., Berndes, G., & Ravindranath, N. H. (2011). Performance of Jatropha biodiesel production and its environmental and socio-economic impacts - A case study in Southern India. *World Renewable Energy Congress*, 2470–2477.

Ayadi, F. Y., Rosentrater, K. A., & Muthukumarappan, K. (2012). Alternative protein sources for aquaculture feeds. *Journal of Aquaculture Feed Science and Nutrition*, *4*(1), 1–26.

Bailey, R. W. (1973). Structural carbohydrates. In G. W. Butler & R. W. Bailey (Eds.). Chemistry and Biochemistry of Herbage (Vol. 1, pp. 157–211). New York: Academic Press.

Becker, K., & Makkar, H. P. S. (1999). Effects of dietary tannic acid and quebracho tannin on growth performance and metabolic rates of common carp (*Cyprinus carpio* L.). *Aquaculture*, *175*, 327–335.

Becker, K., Wulfmeyer, V., Berger, T., Gebel, J., & Münch, W. (2013). Carbon farming in hot, dry coastal areas: an option for climate change mitigation. *Earth System Dynamics*, *4*(2), 237–251.

Borman, G. D., von Maltitz, G. P., Tiwari, S., & Scholes, M. C. (2013). Modelling the economic returns to labour for *Jatropha* cultivation in southern Africa and India at different local fuel prices. *Biomass and Bioenergy*, *59*, 70–83.

Bureau, D. P., Harris, A. M., & Cho, C. Y. (1998). The effects of purified alcohol extracts from soy products on feed intake and growth of chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *161*, 27–43.

Cao, L., Yang, Y., Wang, W. M., Yakupitiyage, a., Yuan, D. R., & Diana, J. S. (2008). Effects of pretreatment with microbial phytase on phosphorous utilization and growth performance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture Nutrition*, *14*(2), 99–109.

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.

Choct, M., Hughes, R. J., Wang, J., Bedford, M. R., Morgan, A. J., & Annison, G. (1996). Increased small intestinal fermentation is partly responsible for the anti- nutritive activity of non-starch polysaccharides in chickens. *British Poultry Science, 37*, 609–921.

De, D.K., Nath, D., Sen, P.R. (1987). Preliminary studies on tea seed-cake as a fish toxicant. *Indian Journal of Animal Science, 57*(7), 781–783.

Debnath, D., Pal, A. K., Sahu, N. P., Jain, K. K., Yengkokpam, S., & Mukherjee, S. C. (2005). Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. *Aquaculture Research, 36*, 180-187.

De Lange, C. F. M. (2000). Characterisation of the non-starch polysaccharides. In P. J. Moughan, M. W. A. Verstegen, & M. I. Visser-Reyneveld (Eds.), Feed evaluation principles and practice (pp. 77–92). The Netherlands: Wageningen Pers, Wageningen.

Eeckhout, W., & De Paepe, M. (1994). Total phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs. *Animal Feed Science and Technology*, *47*(1-2), 19–29.

FAO Fisheries and Aquaculture. (2011). *Demand and supply of feed ingredients for farmed fish and crustaceans* (p. 87).

FAO Fisheries and Aquaculture Department. (2012). *World Review of Fisheries and Aquaculture, Part 1* (p. 104 pp).

FAO Fisheries and Aquaculture Department. (2013). Global aquaculture production statistics for the year 2011. *ftp://ftp.fao.org/FI/news/GlobalAquacultureProductionStatistics2011.pdf*.

Fox, S. W., & Davies, S. J. (2011). Corn-expressed dietary phytase augments vertebral and scale mineral content in juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Nutrition*, *17*(4), e840-e852.

Francis, G., Makkar, H. P. S., & Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, *199*(3-4), 197–227.

Franceschi, V. R., & Nakata, P. A. (2005). Calcium oxalate in plants: formation and function. *Annual Review of Plant Biology*, *56*, 41–71.

Gatlin, D. M., Barrows, F. T., Brown, P., Dabrowski, K., Gaylord, T. G., Hardy, R. W., … Wurtele, E. (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Research*, *38*(6), 551–579.

Goel, G., Makkar, H. P. S., Francis, G., & Becker, K. (2007). Phorbol esters: structure, biological activity, and toxicity in animals. *International Journal of Toxicology*, *26*(4), 279–288.

Grant, G. (1991). Lectins. *D'Mello, F.J.P., Duffus, C.M., Duffus, J.H. Eds. Toxic Substances in Crop Plants*. The Royal Society of Chemistry, Thomas Graham House, Science Park, Cambridge CB4 4WF, Cambridge, pp. 49–67.

Guedes, R. E., Cruz, F. D. A., Lima, M. C. De, Sant'Ana, L. D., Castro, R. N., & Mendes, M. F. (2014). Detoxification of *Jatropha curcas* seed cake using chemical treatment: Analysis with a central composite rotatable design. *Industrial Crops and Products*, *52*, 537–543.

Hardy, R. W. (2010). Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Aquaculture Research*, *41*(5), 770-776.

Hart, S. D., Bharadwaj, A. S., & Brown, P. B. (2010). Soybean lectins and trypsin inhibitors, but not oligosaccharides or the interactions of factors, impact weight gain of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *306*(1-4), 310–314.

Hopwood, D. E., Pethick, D. W., & Hampson, D. J. (2002). Increasing the viscosity of the intestinal contents stimulates proliferation of enterotoxigenic *Escherichia coli* and *Brachyspira pilosicoli* in weaner pigs. *The British Journal of Nutrition*, *88*(5), 523-532.

Jain, S., & Sharma, M. P. (2010). Prospects of biodiesel from *Jatropha* in India: A review. *Renewable and Sustainable Energy Reviews*, *14*(2), 763–771.

Kesava Rao, A. V. R., Wani, S. P., Singh, P., Srinivas, K., & Srinivasa Rao, C. (2012). Water requirement and use by *Jatropha curcas* in a semi-arid tropical location. *Biomass and Bioenergy*, *39*, 175–181.

Koh, M. Y., & Mohd. Ghazi, T. I. (2011). A review of biodiesel production from *Jatropha curcas* L. oil. *Renewable and Sustainable Energy Reviews*, *15*(5), 2240–2251.

Kongmany, S., Matsuura, H., Furuta, M., Okuda, S., Imamura, K., & Maeda, Y. (2013). Plasma application for detoxification of *Jatropha* phorbol esters. *Journal of Physics: Conference Series*, *441*, 1–6.

Kumar, A., & Sharma, S. (2008). An evaluation of multipurpose oil seed crop for industrial uses (*Jatropha curcas* L.): A review. *Industrial Crops and Products*, *28*(1), 1–10.

Kumar, V., Makkar, H. P. S., & Becker, K. (2010). Dietary inclusion of detoxified *Jatropha curcas* kernel meal: effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L. *Fish Physiology and Biochemistry*, *36*(4), 1159-1170.

Kumar, V., Makkar, H. P. S., Devappa, R. K., & Becker, K. (2011a). Isolation of phytate from *Jatropha curcas* kernel meal and effects of isolated phytate on growth, digestive physiology and metabolic changes in Nile tilapia (*Oreochromis niloticus* L.). *Food and Chemical Toxicology*, *49*(9), 2144–2156.

Kumar, V., Makkar, H. P. S., & Becker, K. (2011b). Detoxified *Jatropha curcas* kernel meal as a dietary protein source: growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings. *Aquaculture Nutrition*, *17*(3), 313-326.

Kumar, V., Makkar, H. P. S., & Becker, K. (2011c). Nutritional, physiological and haematological responses in rainbow trout (*Oncorhynchus mykiss*) juveniles fed detoxified *Jatropha curcas* kernel meal. *Aquaculture Nutrition*, *17*(4), 451–467.

Kumar, V., Akinleye, A. O., Makkar, H. P. S., Angulo-Escalante, M. A., & Becker, K. (2012a). Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing *Jatropha platyphylla* kernel meal as a protein source. *Journal of Animal Physiology and Animal Nutrition*, *96*(1), 37–46.

Kumar, V., Sinha, A. K., Makkar, H. P. S., De Boeck, G., & Becker, K. (2012b). Phytate and phytase in fish nutrition. *Journal of Animal Physiology and Animal Nutrition*, *96*(3), 335–364.

Leenhouwers, J. I., Verreth, J. A. J., & Schrama, J. W. (2006). Digesta viscosity, nutrient digestibility and organ weights in African catfish (*Clarias gariepinus*) fed diets supplemented with different levels of a soluble non-starch polysaccharide. *Aquaculture Nutrition*, (12), 111-116.

Libert, B., & Franceschi, V. R. (1987). Oxalate in crop plants. *Journal of Agricultural and Food Chemistry*, *35*(6), 926–938.

Liebert, F., & Portz, L. (2005). Nutrient utilization of Nile tilapia fed plant based low phosphorus diets supplemented with graded levels of different sources of microbial phytase. *Aquaculture*, *248*(1-4), 111-119.

Makkar, H.P.S. (1993). Antinutritional factors in foods for livestock. *Gill, M., Owen, E., Pollot, G.E., Lawrence T.L.J. Eds. Animal Production in Developing Countries. Occasional publication No. 16. British Society of Animal Production*, pp. 69–85.

Makkar, H. P., & Becker, K. (1999). Nutritional studies on rats and fish (carp *Cyprinus carpio*) fed diets containing unheated and heated *Jatropha curcas* meal of a non-toxic provenance. *Plant Foods for Human Nutrition (Dordrecht, Netherlands)*, *53*(3), 183-192.

Makkar, H. P. S., & Becker, K. (2009). *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. *European Journal of Lipid Science and Technology*, *111*(8), 773-787.

Makkar, H., & Becker, K. (2011). Detoxification of *Jatropha curcas* meal for feeding to farm animal species and fish. *United States Patent Application Publication*. US 2011/0311710 A1.

Mosior, M., & Newton, A. C. (1995). Mechanism of interaction of protein kinase C with phorbol esters. *The Journal of Biological Chemistry*, *270*(43), 25526-25533.

Muir, J. F. (2013). Fish, feeds, and food security. *Animal Frontiers*, *3*(1), 28–34.

Noonan, S. C., & Savage, G. P. (1999). Oxalate content of foods and its effect on humans. *Asia Pacific Journal of Clinical Nutrition*, *8*(1), 64–74.

Nwanna, L. C., Kolahsa, M., Eisenreich, R., & Schwarz, F. J. (2007). Pre-treatment of dietary plant feedstuffs with phytase and its effect on growth and mineral concentration in common carp (*Cyprinus carpio* L.). *Journal of Animal Physiology and Animal Nutrition*, *92*(6), 677-682.

NRC 2011: *Nutrient Requirements of Fish and Shrimp*. National Research Council, Washington DC. The National Academic Press. 376 pp.

OECD-FAO. (2011). Agricultural Outlook 2011 - 2020. *OECD Publishing and FAO. http://dx.doi.org/10.1787/agr_outlook-2011-En.*

Olli, J. J., Hjelmeland, K., & Krogdahl, A. (1994). Soybean trypsin inhibitors in diets for Atlantic salmon (*Salmo salar*, L): effects on nutrient digestibilities and trypsin in pyloric caeca homogenate and intestinal content. *Comparative Biochemistry and Physiology. Part A, Physiology*, *109*(4), 923-928.

Olvera-Novoa, M. A., Campos, S. G., Sabido, M. G., & Martínez Palacios, C. A. (1990). The use of alfalfa leaf protein concentrates as a protein source in diets for tilapia (*Oreochromis mossambicus*). *Aquaculture*, *90*(3-4), 291-302.

Olsen, R. L., & Hasan, M. R. (2012). A limited supply of fishmeal: Impact on future increases in global aquaculture production. *Trends in Food Science & Technology*, *27*(2), 120–128.

Openshaw, K. (2000). A review of *Jatropha curcas*: an oil plant of unfulfilled promise. *Biomass & Bioenergy*, *19*, 1–15.

Phengnuam, T. & Suntornsuk, W. (2013). Detoxification and anti-nutrients reduction of *Jatropha curcas* seed cake by *Bacillus* fermentation. *Journal of Bioscience and Bioengineering, 115*(2), 168-172.

Pradhan, S., Naik, S. N., Khan, M. A. I., & Sahoo, P. (2011). Experimental assessment of toxic phytochemicals in *Jatropha curcas*: oil, cake, bio-diesel and glycerol. *Journal of the Science of Food and Agriculture*, *92*(3), 511–519.

Rahman, M. M., Nakagawa, T., Niimi, M., Fukuyama, K., & Kawamura, O. (2011). Effects of feeding oxalate containing grass on intake and the concentrations of some minerals and parathyroid hormone in blood of sheep. *Asian-Australasian Journal of Animal Sciences*, *24*(7), 940–945.

Rahman, M. M., Abdullah, R. B., & Wan Khadijah, W. E. (2013). A review of oxalate poisoning in domestic animals: tolerance and performance aspects. *Journal of Animal Physiology and Animal Nutrition*, *97*(4), 605–614.

Rao, K. V. Rao, T. P. Reddy, & Reddy V. D. (2009). Molecular characterization, physiochemical properties, known and potential applications of phytases: An overview. *Critical Reviews in Biotechnology*, *29*(2), 182-198.

Refstie, Ê., Svihus, B., & Shearer, K. D. (1999). Nutrient digestibility in Atlantic salmon and broiler chickens related to viscosity and non-starch polysaccharide content in different soyabean products. *Animal Feed Science and Technology*, *79*, 331-345.

Riche, M., & Garling, D. L. (2004). Effect of phytic acid on growth and nitrogen retention in tilapia *Oreochromis niloticus* L. *Aquaculture Nutrition*, *10*(6), 389–400.

Riche, M., & Williams, T.N. (2011). Fishmeal replacement with solvent-extracted soybean meal or soy protein isolate in a practical diet formulation for Florida pompano (*Trachinotus carolinus*, L.) reared in low salinity. Aquaculture Nutrition, 17, 369-379.

Sandholm, M., Smith, R. R., Shih, J. C. H., & Scott M. L. (1976). Determination of antitrypsin activity on agar plates: Relationsship between antitrypsin and biological value of soybean for trout. *The Journal of Nutrition, 106*, 761-766.

Sargut, S. T., Sayan, P., & Kiran, B. (2010). Influence of essential and non-essential amino acids on calcium oxalate crystallization. *Crystal Research and Technology*, *45*(1), 31–38.

Savage, G. P., Vanhanen, L., Mason, S. M., & Ross, A. B. (2000). Effect of cooking on the soluble and insoluble oxalate content of some New Zealand foods. *Journal of Food Composition and Analysis*, *13*(3), 201–206.

Singh, S. P., & Singh, D. (2010). Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: A review. *Renewable and Sustainable Energy Reviews*, *14*(1), 200–216.

Sinha, A. K., Kumar, V., Makkar, H. P. S., De Boeck, G., & Becker, K. (2011). Non-starch polysaccharides and their role in fish nutrition – A review. *Food Chemistry*, *127*(4), 1409-1426.

Slawski, H., Adem, H., Tressel, R.-P., Wysujack, K., Koops, U., Wuertz, S., & Schulz, C. (2011). Replacement of fish meal with rapeseed protein concentrate in diets fed to wels catfish (*Silurus glanis* L.). *Aquaculture Nutrition*, *17*(6), 605–612.

Spinelli, J., Houle, C., & Wekell, J. (1983). The effect of phytates on the growth of rainbow trout (*Salmo gairdneri*) fed purified diets containing varying quantities of calcium and magnesium. *Aquaculture*, *30*(1-4), 71-83.

Stadtlander, T. (2012). *Saponin fractions from fenugreek (Trigonella foenum-graecum L.) as dietary supplements for Nile tilapia (Oreochromis niloticus L.) and common carp (Cyprinus carpio L.)*. Ph.D.-Thesis, University of Hohenheim, Germany.

Storebakken, T., Shearer, K. D., & Baeverfjord, G. (2000). Digestibility of macronutrients, energy and amino acids, absorption of elements and absence of intestinal enteritis in Atlantic salmon, *Salmo salar*, fed diets with wheat gluten. *Aquaculture*, *184*(1-2), 115–132.

Tacon, A. G. J., & Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture*, *285*(1-4), 146-158.

van der Ingh, T. S. G. A. M., Krogdahlb, A., & Olli, J. J. (1991). Effects of soybean-containing diets on the proximal and distal intestine in Atlantic salmon (*Salmo salar*): a morphological study. *Aquaculture*, *94*, 297-305.

Vandenberg, G. W., Scott, S. L., Sarker, P. K., Dallaire, V., & de la Noüe, J. (2011). Encapsulation of microbial phytase: Effects on phosphorus bioavailability in rainbow trout (*Oncorhynchus mykiss*). *Animal Feed Science and Technology*, 1-14.

Veerabhadrappa, M. B., Shivakumar, S. B., & Devappa, S. (2013). Solid-state fermentation of *Jatropha* seed cake for optimization of lipase, protease and detoxification of anti-nutrients in Jatropha seed cake using *Aspergillus versicolor* CJS-98. *Journal of Bioscience and Bioengineering*, *117*(2), 208–214.

Vielma, J., Ruohonen, K., Gabaudan, J., & Vogel, K. (2004). Top-spraying soybean meal-based diets with phytase improves protein and mineral digestibilities, but not lysine utilization in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*, *35*(10), 955-964.

Wang, F., Yang, Y.-H., Han, Z.-Z., Dong, H.-W., Yang, C.-H., & Zou, Z.-Y. (2008). Effects of phytase pretreatment of soybean meal and phytase-sprayed in diets on growth, apparent digestibility coefficient and nutrient excretion of rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquaculture International*, *17*(2), 143-157.

Wang, X.-H., Ou, L., Fu, L.-L., Zheng, S., Lou, J.-D., Gomes-Laranjo, J., … Zhang, C. (2013). Detoxification of *Jatropha curcas* kernel cake by a novel *Streptomyces fimicarius* strain. *Journal of Hazardous Materials*, *260*, 238–246.

Wani, S. P., Chander, G., Sahrawat, K. L., Srinivasa Rao, C., Raghvendra, G., Susanna, P., & Pavani, M. (2012). Carbon sequestration and land rehabilitation through *Jatropha curcas* (L.) plantation in degraded lands. *Agriculture, Ecosystems & Environment*, *161*, 112–120.

Wee, K. L., Shu, S. (1989). The nutritive value of boiled full-fat soybean in pelleted feed for Nile tilapia. *Aquaculture, 81*, 303-314.

Weerd, J. H. V. (1999). Balance trials with African catfish *Clarias gariepinus* fed phytase-treated soybean meal-based diets. *Aquaculture Nutrition*, *5*(2), 135-142.

Wilson, R. P., & Poe, W. E. (1985). Effects of feeding soybean meal with varying trypsin inhibitor activities on growth of fingerling channel catfish. *Aquaculture*, *46*, 19-25.

Workagegn, K. B., Ababbo, E. D., & Tossa, B. T. (2013). The effect of dietary inclusion of *Jatropha curcas* kernel meal on growth performance, feed utilization efficiency and survival rate of juvenile Nile tilapia. *Journal of Aquaculture Research & Development*, *4*(5), 1–5.

Yousif, O. M., Alhadhrami, G. A., & Pessaraklib, M. (1994). Evaluation of dehydrated alfalfa and salt bush (*Atriplex*) leaves in diets for tilapia (*Oreochromis aureus* L .). *Aquaculture*, *126*(94), 341–347.

2 General Material and Methods

2.1 Experimental design and set-up

2.1.1 Recirculation systems

All experiments were conducted in recirculation systems. The systems consisted of the experimental tanks in form of glass aquaria or plastic tanks of either 25L or 45L in volume. Total water volume was 2 m^3 and 6 m^3 respectively. Both systems were connected to a sedimentation tank, which allowed particulate organic matter, such as faeces to settle and be siphoned out. Water from the sedimentation tank ran to a sump from which the water was pumped to a bio-filtration tower containing plastic bio-media with nitrifying bacteria. A diagram of the systems used is shown in Figure 2.1.1. Two separate types of bio-filtration were used: A trickling filter or a water-immersed bio-filter. The advantages of a trickling filter imply no need of using additional aeration as oxygen supply is reached by diffusion and splashing of the water. On the contrary, equally-sized immersed bio-filters can clear higher volumes of water compared to trickling filters. Water from the bio-filter either ran back to the sump and from there to the experimental tanks or to another holding tank from where it was then pumped to the experimental tanks. The systems all contained automated temperature control regulation with heaters in the sump. In cases of severe temperature differences between water and surroundings, the room heating system was used to support the water temperature control system. Weekly water quality tests implying ammonia, nitrite, nitrate, pH and dissolved oxygen were conducted and values were archived. In addition to automated temperature control, tank water temperature was measured daily with a thermometer. Lime was added to the sump whenever pH values were lower than 6.5. An air pump ensured oxygenation of the

water in the experimental tanks. General routine during experiments comprised by-weekly cleaning of the tank in- and outflows as well as the tanks themselves. Routine water changes of about 5-10% of the system water were conducted about every 5 days during an experiment. Water flow in the experimental tanks was at least 1.5 L / min.

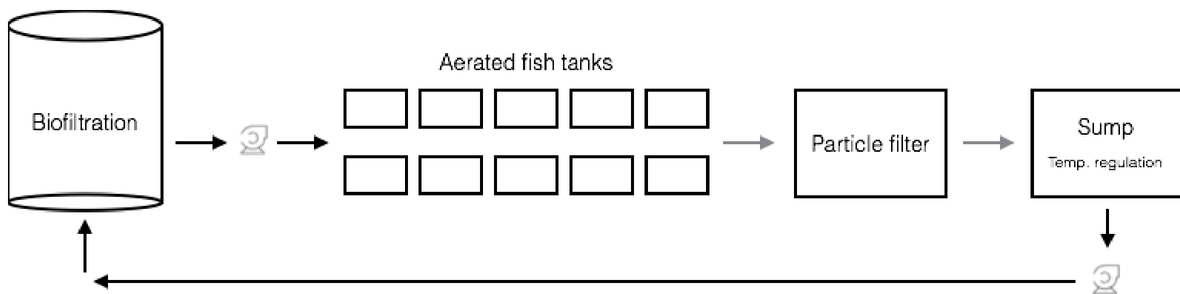


Figure 2.1.1 Diagram of recirculation system used for experiments.

2.1.2 Experimental design

Dependent on the type of experiment, different number of treatments and replicates were chosen. Throughout most trials, the number of fish used per experiment was strongly influenced by the availability of detoxified JKM, which limited the amount of feed possibly produced. Generally, the aim was to run the trials with as many replicates and as many fish per replicate as possible. A minimum of four replicates was kept at all times. The occasional shortage of detoxified JKM especially influenced trials with tilapia as the minimum stocking density to minimize territorial behavior could not be met. The consequence was aggressive behavior within a tank, which in some trials resulted in mortalities. The alternative was to keep fish in single tanks, which was conducted in one trial with n = 8 replicates. Fish initial weight for all trials was between 5-15 g. Within a trial the standard deviation of the initial fish weight was kept as low as possible with the respective stock of fish available. Guidelines from the NRC (2011) recommending a minimum trial period of 8 weeks or an increase in body mass of at least 300% for juvenile fish were adapted.

2.1.3 Feeding schedule

The method of establishing the correct feeding regime was adapted from Richter et al. (2002). The principle is to adapt the amount fed to the fish according to multiples of the basic maintenance ration, which is the amount of feed a fish needs to neither gain nor lose weight. The basic maintenance ration is dependent on the species and the water temperature and can be calculated according to the following formula:

$f = (a / 1000)^{0.8} * b$

Where:

f = amount of feed (g)

a = fish weight (g)

b = metabolic factor ($g / kg^{0.8}$)

At a water temperature of 24°C, b equals 2.83 for tilapia and 3.20 for carp, showing that carp has a higher maintenance requirement of feed at the same temperature than tilapia, provided the feed has equal protein and energy content as well as equal nutrient availabilities.

When evaluating a test diet for nutrient availability, complete ingestion of a given feed ration by experimental fish, is of key importance. If differences in feed ingestion between treatments were the case, differences in fish development could not be solely attributed to diet formulation making an analysis of nutrient availability impossible. Therefore, experimental fish during a trial were given the maximum amount of feed consumed completely. This was usually a maximum of 5 times the maintenance ration. During the first days of an experiment, fish were fed lower multiples of the maintenance ration, which were steadily increased over the next days to adapt fish to increased feeding. Every two weeks fish were weighted, the new maintenance ration determined and feed amounts adjusted.

2.2 Production of experimental diets

Production of experimental diets was generally performed in the same manner for all trials. The bulk ingredients (meals, cellulose) were mixed in a standard kitchen blender first, before the ingredients with lower dietary concentrations were added (mineral and vitamin premixes, crystalline amino acids, digestibility markers, organic acids / salts). Ingredients with less than 2% content in the diet were first hand-mixed with a small portion of the mix, then further hand-mixed to a larger portion before added to the kitchen blender, thereby ensuring optimal distribution of the respective ingredient in the final mix. After mixing, oil and water were added. The amount of water added depended on the type of raw material used for the feed and was between 30% – 50%. Feed moisture was optimized for a mincer, which processed the dough to noodles. If feed additives in form of liquid enzymes were added, they were mixed to the water beforehand. Diet processing in the mincer implied undesired heating of the dough through friction. Whenever heat labile substances such as enzymes were added to the diet, it was ensured that temperature did not exceed 50°C by cooling the barrel of the mincer. After mincing, the noodles were chopped to small pieces, laid out on aluminum foil and dried either in a drying chamber or in a heated, well-ventilated room at 45°C for a minimum of 48 hours. After drying diets were stored either at -20°C in plastic bags until trial start (NRC, 2011).

2.3 Pre- and post-experimental activities

Fish were put in the trial recirculation system at least one week in advance to trial start. The system temperature was adapted to the temperature fish had been exposed to before and if this temperature varied from the trial temperature, it was steadily increased over the following days. At the day of experiment start, fish were weighed into the tanks and at least 8 replicate starting fish were sacrificed with ethylenglycolmonophenylether (5 ml / L). The same method was applied to sacrifice

fish after the experiment. Processing of experimental fish was always according to the same procedure: Dead fish of one replicate tank were transferred into a 1 L glass beaker with 200 ml distilled water added. They were autoclaved at 120°C for 15 minutes before the fish slurry was homogenized with an ultra turrax blending device and quantitatively transferred into a pre-weighed, numbered, 500 ml plastic container. The container was closed with a perforated cover and frozen at -20°C for at least 16 hours. Subsequently, the containers were freeze-dried. Complete dryness was reached when the temperature of the drying chamber of the freeze-dryer reached room temperature and no increase in pressure of the drying chamber could be observed when the connection of sample containers to the freezing element was interrupted. After drying, containers were weighed again and samples homogenized in an analytical grinder. Closed lids were applied for long-term storage of material at 4°C.

For those experiments where blood parameters were measured, syringes were heparin treated one day before the experiment and openly stored at 4°C over night in order for remaining water in the syringe to evaporate. Fish were punctured immediately after sacrifice in the caudal vein and blood was transferred in an Eppendorf tube, which was immediately centrifuged at 4000 rpm at room temperature. Supernatant plasma was pipetted to another Eppendorf tube which was stored at -20°C until analysis. Plasma enzymes were not measured; therefore lower storage temperatures were not required.

2.4 Chemical and biochemical analyses

2.4.1 Dry matter

Fish moisture was determined by deducting dried fish matter in the pre-weighed container after freeze-drying from the final fresh weight of the fish taken the last day of the experiment.

Analytical dry matter of feed and tissue material was determined by evaporation of water from material kept in pre-weighed glass containers without lid in a heating chamber at 105°C for one hour.

2.4.2 Lipid

Automated Solvent Extraction (ASE)

An ASE system (Dionex 350) was the preferred method of choice as it proved more practical compared to non-automated methods, (e.g. the method developed by Smedes et al., 1999). Analysis was conducted at the Thuenen Institute of Fisheries Ecology, Hamburg, Germany. About 1 g of dried, homogenized sample was weighed into a glass beaker containing about 1 g of sterilized quartz sand. The sample was mixed with the sand and transferred to stainless steel extraction cells, which were subsequently placed in the ASE system. Extraction in the cells took place under additional heat and pressure (10 MPa) with hexane as the solvent. Solvent containing extracted lipids was collected in a pre-weighed glass container and subsequently evaporated in a rotary evaporator. The glass container was then re-weighed and fat contents determined by deducting the initial, empty weight of the glass container from its final weight.

Soxhlet extraction

Soxhlet extraction of samples was conducted on those trials where ASE was not available (Institute of Aquaculture, Stirling, UK). About 1 g of sample was weighed

into glass tubes with 1 g of Celite and hydrolyzed with 100 ml of boiling 3 M hydrochloric acid for one hour to liberate fat from matrix components in the sample. Afterwards, the acid/sample mix was diluted with 100 ml of deionised water and poured through a filter application. The tube was washed five times with 50 ml of deionised water of approximately 50°C to assure complete transfer of the sample. The hydrolyzed sample remained in the filter, while the acid along with the water was washed out. The filtrate was dried over night at 60°C. Dried filtrate is inserted in the Soxhlet system. For each sample, one extraction cup is pre-weighed. 80 ml of petroleum ether (solvent) is added to the extraction cup. The solvent is heated, evaporates, rises and becomes cooled by a cooling spiral attached to flow-through, cooling water. This condenses the solvent and it trickles down to the filter containing the sample. Lipids are dissolved in the solvent and run through the filter in the extraction cup, where the solvent is re-evaporated. This cycle is repeated several times. The benefit of the Soxhlet system is the possibility of re-using the solvent several times allowing small quantities of solvent per analysis. After complete extraction, the solvent is evaporated at 105°C under a hood and the extraction cup is re-weighed for determining the amount of lipids in the sample.

2.4.3 Ash

Ash content of dried fish was determined by incinerating the sample in pre-weighed ceramic bowls for 1 hour at 550°C in a muffle furnace. The furnace was set to reach its incineration temperature in slow increments over the period of 1.5 hours. Samples were cooled down for one hour in an exsiccator before reweighed and the final weight deducted from the initial weight of the bowl to determine ash content.

2.4.4 Protein

Protein analysis was conducted by measuring sample nitrogen content and multiplying the results by 6.25, which is the average molar ratio of other elements to nitrogen in protein.

True Spec Elemental Analyzer

A True Spec Elemetal Analyzer was used at the Thuenen Institute of Fisheries Ecology in Hamburg, Germany, to analyze nitrogen of sample sizes 100 mg and higher.

Perkin Elmer Elemental Analyzer

For nitrogen analysis of faeces, where only small amounts were available, a 2400 Series 2 Elemental Analyser by Perkin Elmer at the Institute of Aquaculture, Stirling, UK, was used. The instrument provides analysis of sample carbon, hydrogen and nitrogen content. The method allows a sample weight of as little as 2 - 3 mg. The sample is weight into a tin foil weighing boat, which is placed in an auto sampler. The material is mineralized at around 1000°C in an all-oxygen atmosphere resulting in completely oxidized material CO_2, H_2O and NO_x. These gases pass a copper reduction section in silica tube binding remaining oxygen and reducing NO_x to N_2 for subsequent analysis. CO_2, H_2O and N_2 are then quantitatively analyzed in a gas chromatograph with pure helium as carrier gas.

Semi-automated Kjeldahl

Nitrogen samples of some trials were determined through semi-automated Kjeldahl-analysis (Tecator Kjeltec System) at the Institute of Aquaculture, Stirling, UK. In this analysis, the sample is boiled in concentrated sulphuric acid along with a catalyzer (copper sulphate) to convert all organic nitrogen to ammonium sulphate. The ammonium salt is then converted to ammonia by addition of NaOH. The amount of ammonia present (and therefore the amount of nitrogen) is determined through back

titration with boric acid: Ammonia reacts with a certain quantity of boric acid to ammonium borate changing the color of a pH indicator. The machine detects the change in color and adds hydrochloric acid until the indicator adapts its original color again. The amount of hydrochloric acid needed to bring back the original color is proportional to the amount of ammonium borate and consequently nitrogen in the sample.

2.4.5 Energy

Gross energy content of samples was determined through bomb calorimetry with a Parr 6200 bomb calorimeter at the Institute of Aquaculture, Stirling, UK. About 1 g of sample is pelleted using a hand-held pelleter and weighed into a crucible. The crucible is placed into the bomb and a wire connected to conductors is attached above the sample. The bomb is filled with pure oxygen and placed into a bucket filled with exactly 2812.8 g of distilled water. Two electrodes are connected to the bomb. An electrical current is sent through the electrodes and the wire over the sample. When a certain wire temperature is reached, an explosion is caused due to the high reactivity of pure oxygen in the bomb combusting the sample material. Heat, proportional to the energy content of the sample, is emitted and leads to an increase in water temperature. A thermometer detects the increase and calculates it back to kJ / g of sample used. Benzoic acid is used as a standard. Standards contained 26.5 kJ / g and a standard deviation within ± 0.10 was considered acceptable.

2.4.6 Minerals

Mineral analysis of feed, whole body and faeces was conducted at the Institute of Aquaculture, Stirling, UK. Between 20 and 200 mg of sample were weighed in and topped up with 5 ml of concentrated salpetric acid. The material was digested in a CEM Mars Xpress microwave digester for 20 minutes at 190°C. Each sample was then filled up to 25 ml with distilled water. 400 µL of the solution was then further

diluted to 10 ml with distilled water. Samples were stored in the fridge before analyzed in a Thermo Scientific Series 2 Inductively Coupled Plasma Mass Spectrometer (ICP-MS).

2.4.7 Digestibility analysis

For digestibility analysis, an indigestible marker was added to the diet, either Yttrium oxide (Y_2O_3) at 0.1% - 0.5% or titanium dioxide (TiO_2) at 1%. Proximate TiO_2-analysis showed intereference with an unknown component in JKM, which is why results with this method had to be discarded.

Faeces were collected during the last two weeks of an experiment. For tilapia, tanks were cleaned in the morning through siphoning out remaining faeces that accumulated over the past night. Faeces for digestibility analysis were then collected in the late afternoons. For carp, fish were removed from the tank individually and through slight pressure application on the hindgut, faeces were squeezed out. Faeces of one replicate collected over time were pooled in one container. After freeze-drying and homogenising, the Y_2O_3 content was analyzed identitically to the mineral analysis described above.

2.4.8 Blood plasma analysis

Blood plasma parameters were determined in a Fujifilm Dri-Chem NX500i at the Thuenen Institute of Fisheries Ecology, Hamburg, Germany (Kim, 2011). Stored, frozen plasma was thawed to room temperature and, dependent on quantity of analyzed parameters, between 50 and 200 µl were transferred in a specific Fujifilm Eppendorf tube. Parameter analysis is fully automated through a sequence of colorimetric (general chemistry) or potentiometric (sodium, potassium, chloride) reactions.

2.4.9 Phytate

See chapter 4.3

2.4.10 Calculations and equations

Fish growth was expressed through several parameters:

Body mass gain (BMG)

BMG (g) = final body mass in g – initial body mass

BMG (%) = (final body mass in g – initial body mass in g) / initial body mass in g x 100

Specific growth rate (% / day)

SGR = [(ln final body mass in g) – (ln initial body mass in g) / number of trial days] x 100

The specific growth rate assumes an exponential growth of fish over the whole life cycle, which is incorrect. Fish growth rate is weight dependent and therefore SGR will vary over different life stages of fish (i.e. lower with increasing weight). Therefore SGR may not be a suitable parameter in comparing growth of fish between different studies (Dumas et al., 2010). In the present thesis, experimental fish initial weight was always between 5 – 15 g and therefore SGR was considered a valid parameter to assess growth.

Metabolic growth rate (g x kg$^{0.8}$ day^{-1})

MGR = (Body mass gain in g) / [{(initial body mass in g / 1000)$^{0.8}$ + (final body mass in g / 1000)$^{0.8}$} / 2] / number of trial days

The metabolic growth rate can be applied to compare growth between fish fed according to multiples of their metabolic maintenance ration. The exponent corrects for a relatively lower maintenance requirement with increasing body weight of fish. MGR therefore allows for growth rate comparison of fish of different size classes.

Feed conversion ratio (FCR)

FCR = Dry feed fed (g) / body mass gain (g)

Feed conversion is the overall sum of nutrients incorporated into tissue for weight gain. It does not distinguish between nutrients. FCR depends on diet composition, feed quantity, feeding frequency, age and species.

Protein efficiency ratio (PER)

PER = Fresh body mass gain in g / crude protein fed in g

The PER is a measure for the efficiency of dietary protein. Two isoproteic diets resulting in different BMG of experimental fish will yield more or less efficient utilization of protein for growth. However, it does not correct for additional growth coming from other sources (e.g. additional deposition of fat tissue).

Nutrient, mineral and energy retention

Protein productive value, PPV (%) = [(Final fish body protein in g – initial fish protein in g) / total protein consumed in g] x 100

Lipid productive value, LPV (%) = [(final fish body lipid in g – initial fish body lipid in g) / total crude lipid consumed in g] x 100

Mineral retention (%) = [(final fish body mineral content in g – initial fish body mineral content in g) / total amount of mineral consumed in g] x 100

Energy productive value, EPV (%) = [(final fish body energy – initial fish body energy) / (gross energy intake)] x 100

Nutrient retention gives a more precise measure of type of nutrients incorporated in tissue during growth. Normally, protein deposition for muscle growth is the preferred form of weight gain (NRC, 2011).

Nutrient load

N load (g N / kg fish) = (N fed (g) - N deposited (g)) / weight gain (kg).

Nutrient load measures the pollution of effluent water through a nutrient in relation to weight gain.

Apparent dry matter and nutrient digestibility

Apparent dry matter digestibility (%) = 100 * (1 – marker in feed / marker in faeces)

Apparent nutrient digestibility (%) = 100 * (1- (marker concentration in feed / marker concentration in faeces) * (nutrient concentration in faeces / nutrient concentration in feed))

Apparent digestibility is a measure for the availability of a nutrient for absorption in the digestive tract (Kaushik, 1995). Because quantitative faeces collection in fish is impossible, indigestible markers are added to the feed at low concentrations. The present work uses yttrium oxide (Y_2O_3) as a marker. Apparent digestibility is measured under the postulate that faeces consist of nothing but unabsorbed feed. It does not account for substances secreted in the gut that form part of faeces, such as digestive enzymes or minerals. True digestibility analysis would require quantification of the endogenous nutrient losses, which could not be determined in this thesis. However, true and apparent digestibility at least for protein is thought to be similar as long as fish have a high feed intake (NRC, 2011).

2.4.11 Statistical analysis

All statistical calculations are conducted using Statistica 8.0 software (Stat Soft Inc., Tulsa, OK, USA). Data sets are first compiled and if necessary transformed in Microsoft Excel 2007. For all tests the significance level chosen is 5% ($p \leq 0.05$).

The arithmetic mean along with the standard deviation is chosen for the presentation of data.

To test whether samples are distributed normally (i.e. whether the cumulative distribution was significantly different to an ideal Gaussian distribution); Shapiro-Wilk test is applied. Where data is normally distributed, parametric testing is employed to test for significant differences between treatments. A significant Shapiro-Wilk test result ($p \leq 0.05$), means non-parametric testing was employed (Chapter 5.2).

Parametric testing is conducted by applying one-way analysis of variance (ANOVA), which tests for significant differences among treatments for a parameter or dependent variable (e.g. SGR). Each sample is allocated to a specific treatment or categorical predictor (e.g. substitution level of JKM). In the case of two independent variables deployed in one trial (e.g. substitution level of JKM with and without organic acid addition), factorial ANOVA is used allowing multiple categorical predictors to be compared.

In the case of significant differences, either Fisher LSD (Chapter 3.1) or Tukey post-hoc test is applied to determine which treatments differed. In some trials, Dunnett test is applied to test for differences of treatments verses the control.

For not normally distributed data sets, Kruskal-Wallis test is used to determine significant differences between treatments (Chapter 5.2).

Linear regression analysis is conducted to test for significant trends between inclusion levels of dietary components. This allows correlating different concentrations of an ingredient (e.g. soluble oxalate, Chapter 5) to an analyzed parameter (e.g. SGR). R^2 is given as a coefficient of determination where linear regression was employed.

2.5 References

Dumas, A., France, J., & Bureau, D. (2010). Modelling growth and body composition in fish nutrition: where have we been and where are we going? *Aquaculture Research*, *41*(2), 161–181.

Kaushik, S. J., Cravedi, J. P., Lalles, J. P., Sumpter, J., Fauconneau, B., & Laroche, M. (1995). Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, *133*(3-4), 257–274.

Kim, K. H., Lohakare, J. D., Sen, S., Ingale, S. L., Singh, N. K., Park, C. K., … Chae, B. J. (2011). Comparison of growth performance, nutrient digestibility, blood metabolites

and carcass traits in Korean native and crossbred weaning piglets. *Animal Nutrition and Feed Technology*, *11*(2), 159–167.

NRC 2011: *Nutrient Requirements of Fish and Shrimp*. National Research Council, Washington DC. The National Academic Press. 376 pp.

Richter, H., Francis, G., & Becker, K. (2002). A reassessment of the maintenance ration of red tilapia. *Aquaculture International*, *10*(1), 1–9.

Smedes, F. (1999). Determination of total lipid using non-chlorinated solvents. *The Analyst*, *124*(11), 1711–1718.

3 Inclusion of *Jatropha curcas* Kernel Meal in Fishmeal-based and Practical Diets for Tilapia and Carp

3.1 Phytase-incubated *Jatrophas curcas* kernel meal in diets for carp (*Cyprinus carpio*)

August – November 2011

Carsten Krome[1,2], Kim Jauncey[1,2], Sybille Fedderke[1], Ulfert Focken[1]

[1)] Thuenen-Institute of Fisheries Ecology, Ahrensburg, Germany

[2)] Institute of Aquaculture, Stirling, U.K.

Abstract

An experiment was conducted to evaluate the quality of detoxified *Jatropha curcas* kernel meal (JKM) as a protein source on growth and body composition of common carp (*Cyprinus carpio*). Five isonitrogenous and isoenergetic diets were produced: A fishmeal based control diet (diet Control), two diets replacing 50% and 100% of fishmeal protein with JKM (diets J50 and J100) and two diets with equal replacement levels where JKM was pre-incubated with 2000 U / kg phytase for 16 hours beforehand (diets J50Inc and J100Inc). Diets were fed to four replicate tanks per treatment, each containing five carp and attached to a recirculation system for eight weeks. Results showed phytate to be significantly reduced in feeds preincubated with phytase. Fish fed the experimental diets showed no difference in body mass gain, specific growth rate or feed conversion ratio compared to the control at the 50% replacement levels, but significantly worse growth parameters for diets J100 and J100Inc ($p \leq 05$). Direct comparison between diet J50 and J50Inc showed that J50Inc had significantly better body mass gain than J50 ($p = 0.034$). Body lipid content and lipid productive value was significantly higher in the control fish than all other treatments.

In conclusion, JKM can replace 50% of fishmeal protein in diets for carp. Prior phytase-incubation has a positive impact on growth parameters at 50% replacement level, but not at 100%. JKM should further be explored as a protein source in carp diets.

3.1.1 Introduction

In the present article, phytate concentration of JKM is measured and it is documented how pretreatment of JKM-based diets with a commercially available phytase can be applied to reduce phytate content in the diets. In a feeding trial, the pretreated JKM is then tested as a protein source in fish feed for carp. It is examined whether phytase pretreated JKM-based feed replacing fishmeal protein by 50% and 100%, respectively, has an effect on growth parameters of carp compared to non-phytase treated JKM and a fishmeal-based control feed.

3.1.2 Material and methods

Experimental Diets

JKM was obtained from the University of Hohenheim, Germany. Herring meal was obtained from ProEn GmbH, Soltau, Germany. Citric acid was added to the JKM at 11% of its weight. The resulting pH of 4.5 was neutralized to pH 6.0 (resembling the fishmeal pH) with 0.1M NaOH either after incubation of JKM for diets J100Inc and J50Inc or in a separate step in non-phytase, non-incubated diets J100 and J50.

The phytase used was Ronozyme P (DSM), a type isolated from the fungus *Peniophora lycii*. Pre-trials showed unacidified JKM not to react to incubation with phytase no matter how high the activity. It was shown that this was due to the basic nature of the meal. Acidifying with citric acid to pH 4.5 was applied as this was the ideal pH for the phytase-type used (Rao et al., 2009). Phytase was added at 2000 U / kg and incubation took place at 45°C for 16 hours (Cain and Garling, 1995).

The fishmeal diet was mixed to obtain 38% digestible protein, 10% crude lipid, 10% crude ash and 6% crude fiber. The test diets were isonitrogenous and isoenergetic. Lysine and methionine were added in *Jatropha*-mixed diets to account for the lack of these amino acids in the meal. A vitamin and mineral premix was added at 2% each. The constituents were mixed with a common kitchen blender. 40% water was added so that the diet had a mushy consistency. Subsequently the diet was run through a meat mincer and crumbled into small pieces before dried at 40°C for 24 hours in a drying chamber.

5 diets were produced (Table 3.1.1):

1. A control diet with fishmeal (Control).
2. A diet in which 50% of the fishmeal was isonitrogenously replaced by JKM (J50).
3. A diet in which 50% of the fishmeal was isonitrogenously replaced by phytase-incubated JKM (J50Inc).
4. A diet in which 100% of fishmeal was isonitrogenously replaced by JKM (J100).
5. A diet in which 100% of fishmeal was isonitrogenously replaced by phytase-incubated JKM (J100Inc).

Table 3.1.1 Composition of experimental diets with 50% and 100% fishmeal protein replacement through JKM with and without phytase incubation fed to common carp for 8 weeks.

Ingredient (g/100g)	Control	J100	J100Inc	J50	J50Inc
Fish Meal	49.7	0.0	0.0	24.9	24.9
Wheat Meal	37.3	17.2	17.2	27.3	27.3
JKM (Not incubated)	0.0	67.5	0.0	33.8	0.0
JKM (Incubated)	0.0	0.0	67.5	0.0	33.8
Sun Flower Oil	4.3	4.6	4.6	4.4	4.4
Fish Oil	1.3	5.0	5.0	3.1	3.1
Cellulose	3.4	0.8	0.8	2.1	2.1
Vitamin Premix[a]	2.0	2.0	2.0	2.0	2.0
Mineral Premix[b]	2.0	2.0	2.0	2.0	2.0
Lysine	0.0	0.8	0.8	0.4	0.4
Methionine	0.0	0.1	0.1	0.1	0.1
Total	**100**	**100**	**100**	**100**	**100**

[a]Composition of vitamin premix (mg/kg, unless otherwise stated): Vitamin A: 500,000 I.E./kg; Vitamin D3: 50,000 I.E./kg; Vitamin E: 2500; Vitamin K3 (as Menadione): 1000; Vitamin B1: 5000; Vitamin B2:5000; Vitamin B6: 5000; Vitamin B12: 5; Nicotinic acid: 25000; Pantothenic acid: 10000; Folic acid: 1000; Biotin: 250; Choline chloride: 100000; Inositol: 25000; Vitamin C: 20125.
[b]Composition of mineral premix (supplied by Altromin Spezialfutter GmbH. (mg/kg mix): Calcium: 122,160; Phosphorus: 83,670; Magnesium: 14,960; Sodium: 18,180; Potassium: 210,250; Sulfur: 15,460; Chlorine: 29,720; Iron: 1220; Manganese: 1000; Zinc: 1630; Copper: 155; Iodine: 120.
[c]Ronozyme P (L) by DSM

Table 3.1.2 Proximate composition of experimental diets fed to common carp for 8 weeks.

Diet	Dry Matter	Crude Protein	Crude Lipid	Ash
J100	90.7	40.7	9.3	11.5
J100Inc	91.3	43.1	8.9	11.7
J50	93.2	41.1	10.5	10.7
J50Inc	93.9	42.8	9.6	11.4
Control	92.4	42.8	11.1	10.1

Values in % dry matter

Measurement of phytate

Phytate was measured according to the method of Latta and Eskin (1980) refined by Vaintraub & Lapteva (1988). A sample of 0.5 g of each diet was dissolved in 10 ml 3.5% HCl and stirred for 2h in a magnetic stirrer to extract the phytate from the plant

material. Subsequently, the sludge was centrifuged at 3000 rpm for 10 min. The supernatant was transferred to new tubes and centrifuged again at 10000 rpm for 10 minutes. The remaining supernatant was diluted with water at a 1/5 ratio. 0.1 ml of this solution was added to 1 ml of Wade-reagent (Wade et al., 1953) and 2.9 ml of water and the absorbance was analyzed at 500 nm light stream in the photometer and compared against a standard curve with known phytate concentrations.

Feeding trial

Twenty 60 L tank inhabited 5 fish each and n = 4 tanks formed the number of replicates of each of the 5 treatments. The tanks were set up in a randomized manner. Water flow rate was set to 1.5 L / min and aeration was provided. Fish were fed 4 times a day over a period of 8 weeks. Water temperature was between 22° and 23°C throughout the experiment.

As a parameter for feed quantity, the metabolic maintenance ration of the fish was calculated (see 2.1.3).

Pre- and post experimental procedures as well as performance calculations and equations were conducted as outlined in Chapter 2.

Statistics

One-way ANOVA ($p \leq 0.05$) was used to analyze treatments. Dunnett test was applied to test for significant differences from the control diet for the growth data. Fisher LSD test was used to check differences in body composition within final and between final and initial fish. Student´s t-test was used to test for differences in growth between the incubated and the not incubated diets. Values are shown in mean ± standard deviation.

3.1.3 Results

The phytate content of JKM and the diets after incubation is shown in Table 3.1.3.

Fish behavior

Feed intake of up five times the metabolic body weight was shown to be possible

Table 3.1.3 Phytate contents of experimental diets with and without phytase-incubation fed to common carp for 8 weeks.

Diet	Phytate
J100	4.01
J100Inc	1.33
J50	1.88
J50Inc	0.69
Control	0.00

Values in % DM

Samples measured according to Vaintraub & Lapteva, 1988

without any feed left in the tanks. However, for diet J100Inc, fish needed more time to feed on the diets than fish fed the other diets. Pellets were picked up and spat out several times. Generally, fish fed J100Inc and J50Inc seemed more active than others, never standing in the water, but continuously swimming. The odor of these diets was more acidic compared to the non-incubated feeds. Palatability of J100Inc seemed to be less than the other treatments. Palatability of all other feeds seemed equal. One fish died during the experiment as it jumped out of its tank in week 7 of the experiment.

Growth performance

Figure 3.1.1 shows the development of fish growth over the course of the 8 week experiment. The fish meal based control showed the best growth along with J50Inc. J50 is slightly less, while the J100 and J100Inc are a lot less in growth. Statistical testing for differences from the control (Dunnett test) revealed that only J100 and J100Inc are significantly different from the control, while J50 ($p = 0.072$) and J50Inc ($p = 0.992$) are equal to the control. However, direct comparison of J50 with J50Inc showed there to be a significant difference between the final weights of the fish re-

vealing a difference in quality of the incubated to the not incubated feed (Student´s t-test, $p = 0.034$).

Table 3.1.4 Whole body chemical composition common carp at the start and after fed experimental diets for 8 weeks.

Treatment	Dry Matter		Crude Protein		Crude Lipid		Ash	
Initial Fish	20.9	$\pm1.40^{c}$	72.4	$\pm1.12^{c}$	8.9	$\pm1.19^{d}$	19.3	$\pm1.29^{d}$
J100	23.1	$\pm0.31^{b}$	68.3	$\pm2.83^{b}$	15.7	$\pm3.35^{b,c}$	17.2	$\pm1.83^{c,d}$
J100Inc	23.2	$\pm0.44^{b}$	67.7	$\pm2.78^{b}$	15.8	$\pm0.98^{c}$	16.2	$\pm1.69^{c}$
J50	21.8	$\pm0.38^{c}$	67.8	$\pm1.56^{b}$	18.5	$\pm1.99^{b}$	13.0	$\pm0.92^{b}$
J50Inc	22.2	$\pm0.50^{c}$	65.9	$\pm2.17^{a,b}$	16.6	$\pm1.33^{b,c}$	15.9	$\pm2.29^{c}$
Control	24.0	$\pm0.92^{a}$	63.1	$\pm1.32^{a}$	25.5	$\pm1.45^{a}$	9.4	$\pm0.86^{a}$

Values in % DM
Mean values with different superscript differ significantly from one another, Fisher LSD test ($p \leq 0.05$).

Table 3.1.5 Body mass gain of experimental fish fed different levels of JKM with and without incubation for 8 weeks.

Treatment	IW (g)		FW (g)		BMG (g)		BMG (%)	
J100	12.8	±0.06	26.4	$\pm3.12^{b}$	13.6	$\pm3.09^{b}$	106.4	$\pm23.85^{b}$
J100Inc	12.9	±0.29	25.2	$\pm3.05^{b}$	12.3	$\pm2.95^{b}$	94.8	$\pm22.01^{b}$
J50	13.0	±0.26	37.5	$\pm3.02^{a}$	24.5	$\pm3.00^{a}$	188.1	$\pm23.12^{a}$
J50Inc	12.9	±0.08	44.0	$\pm3.82^{a}$	31.1	$\pm3.77^{a}$	241.2	$\pm28.37^{a}$
Control	12.7	±0.28	44.3	$\pm6.13^{a}$	31.6	$\pm5.91^{a}$	247.5	$\pm41.94^{a}$

Mean values with different superscript differ significantly from one another, Dunnett test ($p \leq 0.05$). Initial weight (IW), final weight (FW), body mass gain (BMG) of experimental fish after fed experimental diets for 8 weeks.

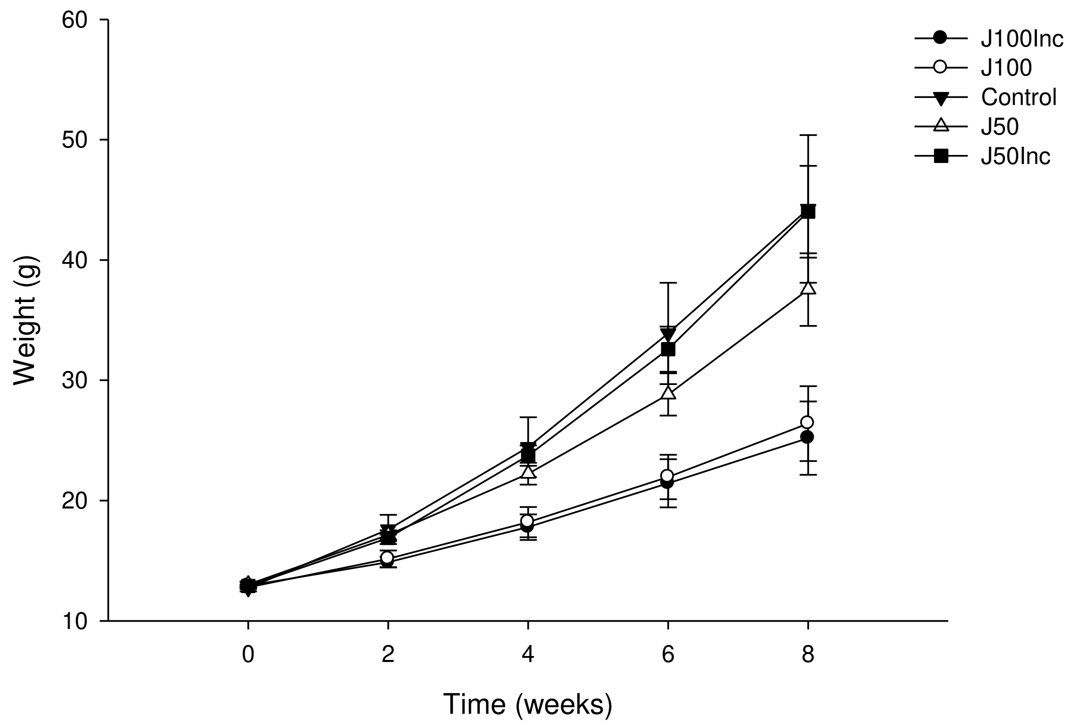


Figure 3.1.1 Growth curves of fish fed different levels of JKM with and without phytase incubation for 8 weeks.

Table 3.1.6 Growth parameters and nutrient utilization of experimental fish fed different levels of JKM with and without phytase incubation for 8 weeks.

Treatment	SGR		FCR		MGR		PER		PPV		LPV	
J100	1.28	±0.21[b]	2.36	±0.51[b]	5.7	±0.98[b]	1.08	±0.20[b]	17.6	±2.39[b]	25.1	±8.39[b]
J100Inc	1.18	±0.21[b]	2.58	±0.57[b]	5.2	±0.95[b]	0.93	±0.19[b]	15.0	±1.76[b]	23.8	±4.72[b]
J50	1.89	±0.15[a]	1.46	±0.15[a]	8.4	±0.67[a]	1.68	±0.16[a]	26.8	±2.29[a]	41.4	±7.68[b]
J50Inc	2.19	±0.15[a]	1.20	±0.12[a]	9.8	±0.69[a]	1.95	±0.19[a]	29.9	±2.51[a]	42.0	±6.04[b]
Control	2.21	±0.22[a]	1.23	±0.16[a]	9.9	±1.05[a]	1.92	±0.23[a]	27.6	±2.85[a]	66.6	±9.08[a]

Data is tested with Dunnett test against the control. Mean values with different superscript differ significantly from one another ($p \leq 0.05$). SGR (% / day), specific growth rate; FCR, feed conversation ratio; MGR (g x $kg^{0.8}$ day^{-1}), metabolic growth rate; PER, protein efficiency ratio; PPV (%), protein productive value; LPV (%), lipid productive value.

The development of the FCR and SGR over the course of the experiment revealed an adaptation process of fish fed diet J50Inc improving both values over time while there was an increase of FCR and decrease of SGR of diet J50. After the first two weeks, there was no statistical difference between J50 and J50Inc (p = 0.746, Student´s t-test), but FCR was significantly lower in J50Inc four weeks into the experiment (p = 0.027) and SGR significantly higher (p = 0.032).

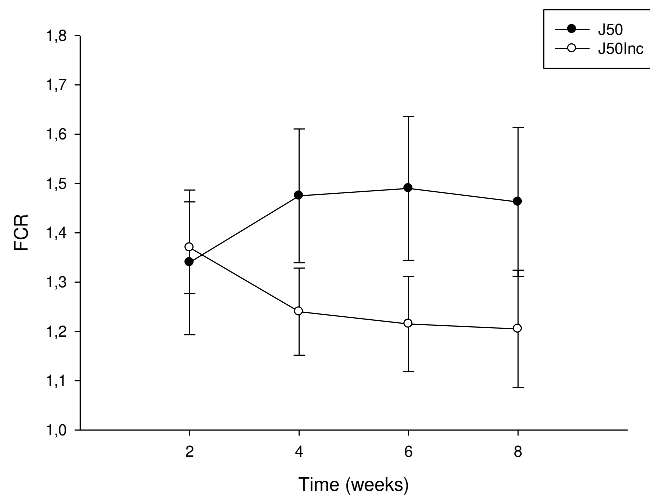


Figure 3.1.2 FCR progression of fish fed 50% of protein through JKM with and without phytase incubation.

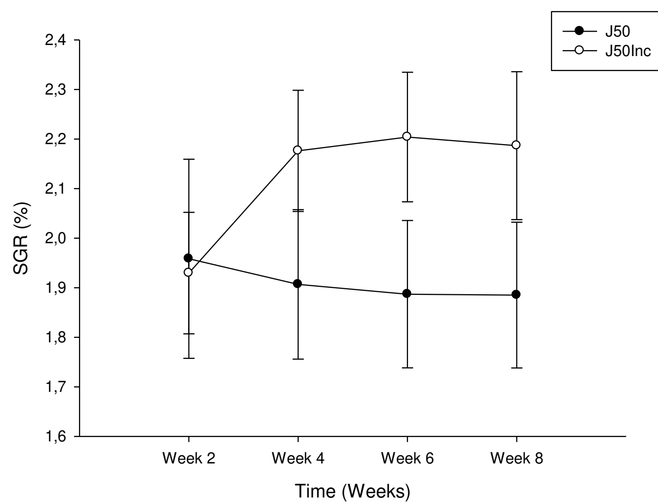


Figure 3.1.3 SGR progression of fish fed 50% of protein through JKM with and without phytase incubation.

3.1.4 Discussion

Pretreatment of JKM

Our results show that the pretreatment process of acidifying and incubating the JKM at 45°C effectively reduces its phytate content to 33.1% (J100) and 36.5% (J50) respectively of the initial value. Incubation with phytase (2900 U / kg) for 1h by Denstadli et al. (2007) was about equally efficient as phytate content was reduced by 66% (9.3 g / kg reduced to 3.2 g / kg) in soy protein concentrate based diets. Cao et al. (2008) pretreated soy meal with a citric acid based buffer with different concentrations of phytase at 55°C for 6h, which reduced phytate concentrations from 4.3 to 0.47 g / kg at activities of 1500 U / kg. Similarly, 1000 U / kg phytase in soybean based diets by Wang et al. (2008) and pretreated in the same process, was enough to eradicate most phytate in these diets.

The motivation of pre-incubating plant feedstuff with phytase to reduce phytate content for carp diets is derived from the fact that carp do not have a stomach and are therefore unable to offer appropriate acidic conditions for the phytase to take effect in the stomach. Furthermore, phytase is also temperature-dependent and usually reaches its highest activities at temperatures above 40°C (Rao et al., 2009), which can also not be provided in a fish tank. Denstadli et al. (2007) compared a 60% soy protein concentrate based, phytase-coated diet with a phytase-incubated diet at 45°C for salmon (*Salmo salar L.*) raised in 8°C and found that the incubated diet had better growth and significantly higher ash and phosphate digestibility. Nwanna et al. (2007) found that phytase-containing pre-incubated plant-based feed resulted in higher growth performance of carp than phytase-containing unincubated feed or the fishmeal control diet. In another experiment by Nwanna et al. (2008) no difference in growth was seen in carp fed diets with and without phytase, both without pretreatment. Pretreatment of diets by spraying the phytase on the diet after pelleting to

circumvent high temperatures in the extrusion process that could lead to denaturing of the enzyme has been applied by Fortes-Silva et al. (2011) for European sea bass. However, no significant weight gain compared to diets without phytase could be documented. Without pretreatment no effect of supplemental phytase on growth was also documented in rainbow trout *(Oncorhynchus mykiss)* (Dalsgaard et al., 2009) or juvenile parrot fish (*Oplegnathus fasciatus*) (Lim et al., 2009). Barua et al. (2007) added phytase and citric acid directly to the diet of Rohu (*Labeo rohita*) and observed significantly better growth than diets with phytase but without citric acid. Similar results were found by Phromkunthong et al. (2010) for carp. In contrast, in another study, rainbow trout showed slightly better growth when fed partially plant-based diets containing phytase compared to ones without phytase (Vandenberg et al., 2011). Still, evidence suggests that pre-treating plant-based diets in conditions aimed to provide an ideal environment for the phytase might be more efficient than just adding the enzyme to the feed. Alternatively, for fish without an acidic stomach, the application of phytase together with citric acid and put directly to the diet without pretreatment may also be an option.

Growth

We observed significantly lower growth in fish fed diets J100 and J100Inc. The consistency of these diets was harder than the ones containing fish meal, which could have changed palatability and digestibility of the diets. Furthermore anti-nutritional factors (apart from phytate) may have hindered assimilation of the diet, in a relatively larger amount compared to J50 and J50Inc. In a trial by Kumar et al. (2011) growth of carp fed a 75% JKM-substituted diet showed slower growth than the 50% substitute and the control. This is contradictory to earlier findings where 75% JKM showed equally good performance, also in carp (Kumar et al., 2010). In the latter trial, JKM showed better values than an equivalent soy protein diet. For trout it was found that the limit of equal performance to a fish meal diet for JKM lies between

50% and 62.5% (Kumar et al., 2010). The results of the present trial show that JKM is a possible substitute for fish meal in carp diets when applied at 50%. Here, neither the incubated (J50Inc) nor the unincubated (J50) diet was statistically different from the control in terms of growth performance. These results are in line with previous findings by Kumar et al. (2011).

Kumar et al. (2011) further reported FCRs of 1.01 ± 0.02 for diets containing 50% JKM. Furthermore even their 75% JKM treatment showed an FCR of 1.21 ± 0.24, both of which are better than what was achieved in the present experiment. However, part of the reason could be that fish in this experiment were around 3.2 ± 0.07 g at the start, whereas fish in this trial had an average starting weight of 12.8 g, thus already further in their development and potentially physiologically not as flexible in adapting to a change in diet compared to younger fish.

When comparing J50 and J50Inc only, there is a significant difference in weight gain and FCR. This shows that while neither diet is statistically different compared to the fishmeal control, the phytase-pretreated diet works better on carp than the one without pretreatment. While the necessity of pretreatment in combination with agastric fish has been discussed, the positive impact of phytase on growth has been documented in carp (Nwanna, 2007) and other fish (Cain and Garling, 1995; Howell et al., 1999; Sajjadi et al., 2004; Debnath et al., 2005). Cao et al. (2008) observed significant growth differences between phytase pretreated and not pretreated diets in Nile tilapia (*Oreochromis niloticus*). These studies used various plant feedstuffs as protein substitutes, however, none of them used JKM. To our knowledge, the present study is the first one to show that phytase pretreated diets lead to faster growth than non-pretreated diets, which have 50% of the fishmeal replaced by JKM.

A clear tendency of the FCR and SGR to improve over time in phytase based diets compared to diets without phytase or the control was also observed. Experimental fish were adapting to the phytase treated diet better than the non-pretreated diet,

which was shown in decreasing FCR-values after 4 weeks in J50Inc verses slightly increasing values for J50 at that time. Slightly increasing FCR values are common with ongoing growth of fish. However, not much information is available about the adaptation process of fish to phytase treated diets or generally plant-based diets. Bonaldo et al. (2006) found a slight decrease in FCR in Egyptian sole (*Solea aegyptiaca*) fed a diet containing 18% soy after 57 days of feeding.

Body composition

Fish fed the control diet showed equal protein productive values compared to the J50 and the J50Inc treatments, but significantly higher lipid productive values. This is reflected in the significantly higher lipid content in the body composition of control fish. High lipid content is commonly regarded as a sign for too much energy or too low protein supply (or protein supply of inadequate amino acid composition) in the diet of the fish. Many authors report high fat content in fish fed plant-based feedstuffs and these have been attributed to the fact that the protein supply of that plant-feed stuff may be of low quality (NRC, 2011). Kumar et al. (2011) show significantly higher lipid content in carp fed a diet replacing 75% of fishmeal with JKM. The present work shows different results as the plant-based diets show significantly less lipid content than the fishmeal fed fish. On an absolute scale, the lipid content of the fish fed JKM-based diets in this trial was also lower than in Kumar et al. (2011) despite higher dietary crude lipid and crude energy content.

In this experiment, phytase inclusion did not have an influence on body composition. This is in line with Sardar et al. (2007) who found no change in crude protein, lipid or ash in juvenile carp body when comparing solely plant-based diets with phytase to the same diet without phytase.

Conclusion

The here documented data shows that JKM incubated with phytase and citric acid results in better growth than the not incubated one at fish meal replacement levels of

50%. JKM could become a useful alternative for fish meal. This counts not only for 50% replacement level, but also potentially for larger fractions. Despite lower growth rates and FCRs at inclusion levels above 50%, a low price of JKM might economically justify its application in commercial diets for carp. With JKM being a promising future source for bio-fuel, chances are good that its by-product will be plentiful and therefore low-priced, thus paving the way for its application in carp diets.

3.1.5 References

Bonaldo, A., Roem, A., Pecchini, A., Grilli, E., & Gatta, P. (2006). Influence of dietary soybean meal levels on growth, feed utilization and gut histology of Egyptian sole (Solea aegyptiaca) juveniles. *Aquaculture*, *261*(2), 580–586.

Cao, L., Yang, Y., Wang, W. M., Yakupitiyage, A, Yuan, D. R., & Diana, J. S. (2008). Effects of pretreatment with microbial phytase on phosphorous utilization and growth performance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture Nutrition*, *14*(2), 99-109.

Dalsgaard, J., Ekmann, K., Pedersen, P., & Verlhac, V. (2009). Effect of supplemented fungal phytase on performance and phosphorus availability by phosphorus-depleted juvenile rainbow trout (*Oncorhynchus mykiss*), and on the magnitude and composition of phosphorus waste output. *Aquaculture*, *286*(1-2), 105-112.

Debnath, D., Pal, A. K., Sahu, N. P., Jain, K. K., Yengkokpam, S., & Mukherjee, S. C. (2005). Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. *Aquaculture Research*, *36*(2), 180-187.

Denstadli, V., Storebakken, T., Svihus, B., & Skrede, A. (2007). A comparison of online phytase pre-treatment of vegetable feed ingredients and phytase coating in diets for Atlantic salmon (*Salmo salar* L.) reared in cold water. *Aquaculture*, *269*(1-4), 414-426.

Fortes-Silva, R., Sánchez-Vázquez, F. J., & Martínez, F. J. (2011). Effects of pretreating a plant based diet with phytase on diet selection and nutrient utilization in European sea bass. *Aquaculture*, *319*(3-4), 417–422.

Kumar, V, Makkar, H P S, & Becker, K. (2010). Dietary inclusion of detoxified *Jatropha curcas* kernel meal: effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L. *Fish Physiology and Biochemistry*, *36*(4), 1159-1170.

Kumar, V., Makkar, H.P.S., & Becker, K. (2011). Detoxified *Jatropha curcas* kernel meal as a dietary protein source: growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings. *Aquaculture Nutrition*, *17*(3), 313-326.

Kumar, V., Akinleye, A. O., Makkar, H. P. S., Angulo-Escalante, M. A, & Becker, K. (2012). Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing *Jatropha platyphylla* kernel meal as a protein source. *Journal of Animal Physiology and Animal Nutrition*, *96*(1), 37–46.

Latta, M; Eskin, M. (1980). A simple and rapid colorimetric method for phytate determination. *Journal of Agricultural and Food Chemistry*, *28*(6), 1313–1315.

Lim, S.-J., & Lee, K.-J. (2009). Partial replacement of fish meal by cottonseed meal and soybean meal with iron and phytase supplementation for parrot fish *Oplegnathus fasciatus*. *Aquaculture*, *290*(3-4), 283-289.

NRC 2011: *Nutrient Requirements of Fish and Shrimp*. National Research Council, Washington DC. The National Academic Press. 376 pp.

Nwanna, L. C., & Schwarz, F. J. (2008). Effect of different levels of phytase on growth and mineral deposition in common carp (*Cyprinus carpio*, L.). *Journal of Applied Ichthyology*, *24*(5), 574-580.

Nwanna, Lawrence C, & Schwarz, Frieder J. (2007). Effect of supplemental phytase on growth, phosphorus digestibility and bone mineralization of common carp (*Cyprinus carpio* L). *Aquaculture Research*, *38*(10), 1037-1044.

Nwanna, L. C., Kolahsa, M., Eisenreich, R., & Schwarz, F. J. (2008). Pre-treatment of dietary plant feedstuffs with phytase and its effect on growth and mineral concentration in common carp (*Cyprinus carpio* L.). *Journal of Animal Physiology and Animal Nutrition*, *92*(6), 677-682.

Phromkunthong, W., Nuntapong, N., & Gabaudan, J. (2010). Interaction of phytase RONOZYME ® P (L) and citric acid on the utilization of phosphorus by common carp (*Cyprinus carpio*). *Songklanakarin Journal of Science and Technology*, *32*(6), 547–554.

Sajjadi, M., & Carter, C. G. (2004). Effect of phytic acid and phytase on feed intake, growth, digestibility and trypsin activity in Atlantic salmon (*Salmo salar* L.). *Aquaculture Nutrition*, *10*(2), 135-142.

Sardar, P., Randhawa, H. S., Abid, M., & Prabhakar, S. K. (2007). Effect of dietary microbial phytase supplementation on growth performance, nutrient utilization, body compositions and haemato-biochemical profiles of *Cyprinus carpio* (L.) fingerlings fed soyprotein-based diet. *Aquaculture Nutrition*, *13*(6), 444–456.

Vaintraub, I. a, & Lapteva, N. a. (1988). Colorimetric determination of phytate in unpurified extracts of seeds and the products of their processing. *Analytical Biochemistry*, *175*(1), 227-230.

Vandenberg, G. W., Scott, S. L., Sarker, P. K., Dallaire, V., & de la Noüe, J. (2011). Encapsulation of microbial phytase: Effects on phosphorus bioavailability in rainbow trout (*Oncorhynchus mykiss*). *Animal Feed Science and Technology*, 1-14.

Wang, F., Yang, Y.-H., Han, Z.-Z., Dong, H.-W., Yang, C.-H., & Zou, Z.-Y. (2008). Effects of phytase pretreatment of soybean meal and phytase-sprayed in diets on growth, apparent digestibility coefficient and nutrient excretion of rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquaculture International*, *17*(2), 143-157.

3.2 Effect of Replacing Different Levels of Dietary Fish Meal with *Jatropha curcas* Kernel Meal on the development of Nile Tilapia (*Oreochromis niloticus*).

January – April, 2012
Journal of Applied Ichthyology (2014), 1-6
Accepted: December 19th, 2013
doi: 10.1111/jai.12414

Carsten Krome[1,2], Kim Jauncey[2], Sibylle Fedderke[1], Ulfert Focken[1]
[1)] Thuenen Institute of Fisheries Ecology, Wulfsdorfer Weg 204, 22926, Ahrensburg, Germany,
[2)] Institute of Aquaculture, University of Stirling, Stirling, UK

Abstract

The present trial tested the applicability of *Jatropha curcas* kernel meal (JKM) as a protein source in diets for Nile tilapia (*Oreochromis niloticus*) in terms of growth and body composition. Four diets were produced replacing 0% (Control), 50% (J50), 75% (J75) and 100% (J100) of fish meal with JKM. A fifth diet, 70% of fishmeal was replaced by JKM, and another 20% were replaced by blood meal to minimize crystalline lysine addition. Growth parameters of fish fed the control diet were better than in all other treatments. However, specific growth rate (SGR) and feed conversion ratio (FCR) was not significantly different between diet J50, J75 and the control. Fish fed the control diet had lower body protein content, but higher body fat and energy content than fish fed the JKM-based diets. Further an adaptation of fish fed diets J50, J75 and J100 could be observed as these diets showed worse FCR-values over most of the first three quarters of the duration of the experiment and equal (or in case of J75 even significantly better) FCR-values over the last two weeks. Despite slightly slower growth, JKM should be further included in the search of alternative plant-feedstuffs in diets for tilapia as growth observed here for up to 75% replacement of fishmeal was very promising.

3.2.1 Introduction

In the present trial, different levels of fishmeal protein of a control diet were replaced by detoxified, defatted JKM. Amino acids were added where necessary to reach the standard requirements of tilapia (NRC, 2011). Replacement levels of 50%, 75%, and 100% of fishmeal protein were tested. Furthermore, an additional diet in which 70% of fishmeal protein was replaced by JKM and another 20% was replaced by blood meal to minimize additional lysine supplementation was tested.

3.2.2 Material and methods

Diet Formulation

JKM was obtained from JatroSolutions GmbH (Stuttgart, Germany). During detoxification of JKM, basic extraction is one of the steps turning the pH of JKM to around 8.5 (Makkar et al., 2011). 1.5% of citric acid was added to JKM to lower the pH of JKM back to neutral. Four experimental diets and a fishmeal based control diet (Diet Control) were designed to be isonitrogenous and isoenergetic. In the test diets, graded levels of fishmeal protein was replaced by JKM in the following amounts: Diet J50 (50% of fishmeal protein replaced by JKM), Diet J75 (75% of fishmeal protein replaced), Diet J100 (100% fishmeal protein replaced) and diet BM (70% of fishmeal protein replaced by JKM, 20% of fishmeal protein replaced by blood meal (supplied by Engelbert Schulz International Trading Ltd.)). Diets were designed to contain 44% crude protein, 10% crude lipid, 10% crude ash and 2% of vitamin and mineral premix respectively. Synthetic amino acids were added to the requirement standards of the NRC (2011) for tilapia in each diet. Phytase (Natuphos 5000 G, BASF) was added to the JKM based diets at a concentration of 2000 U/kg. The constituents were mixed with a household kitchen blender. 40% water was added so that the diet had a mushy consistency. Subsequently the diet was run through a meat mincer and crumbled into small pieces before drying at 40°C for 24 hours in a

ventilated drying chamber. Dietary ingredients and proximate analysis of nutrients are depicted in Table 3.2.1 and Table 3.2.2.

The aim was to provide 40% digestible protein to the experimental fish (Jauncey, 1982). Protein digestibility for fishmeal was assumed at 90.5% (Köprücü and Özdemir, 2005). Data of protein digestibility of JKM for tilapia is not available, referential data was taken from carp at 90.6% (Kumar et al., 2011b).

Experimental animals and setup

The experiment was conducted in a recirculation system. Each of the five dietary treatments was replicated four times. 100 all-male tilapia juveniles, obtained from TilAqua International, Netherlands, 10-15 g were selected from the stock at the Institute of Fisheries Ecology in Ahrensburg, Germany, and randomly distributed at five fish per aquaria. Fish were kept in 45 L glass tanks at 24°C in a recirculation system. Water flow was adjusted to 1.5 l / min. The experiment was set up for 8 weeks.

As a parameter for feed quantity, the metabolic maintenance ration of the fish was calculated (see 2.1.3).

Pre- and post experimental procedures, chemical analyses as well as performance calculations and and equations were conducted as outlined in Chapter 2.

Statistics

One-way ANOVA ($p \leq 0.05$) was used to analyze treatments. Tuckey test was applied as post-hoc test and percentages were arcsine transformed before analysis. FCR development was analyzed with Dunnett test against the control. Statistics were conducted with Statistica 8 software. Values are expressed as mean ± standard deviation.

Table 3.2.1 Composition of experimental diets containing different levels of JKM fed to Nile tilapia for 8 weeks.

Ingredient (g/100g)	Control	J50	J75	J100	BM
Fish Meal	57.1	28.6	14.3	0.0	5.7
Wheat Meal	28.6	21.9	18.6	17.0	24.5
JKM	0.0	34.7	51.1	65.4	43.7
Blood Meal	0.0	0.0	0.0	0.0	11.4
Sun Flower Oil	4.5	4.6	4.6	4.6	4.5
Fish Oil	0.7	2.8	3.9	5.0	4.5
Cellulose	3.5	1.7	0.8	0.4	0.5
Vitamin Premix[1)]	2.0	2.0	2.0	2.0	2.0
Mineral Premix[2)]	2.0	2.0	2.0	2.0	2.0
Histidine	0.55	0.33	0.21	0.10	0.00
Methionine	0.00	0.17	0.34	0.50	0.61
Lysine	0.00	0.39	1.28	2.15	0.53
Threonine	0.29	0.24	0.21	0.18	0.00
Valine	0.51	0.58	0.62	0.65	0.00
Phenylalanine	0.24	0.00	0.00	0.00	0.00

Values based on fresh weight

[1)] Composition (mg/kg, unless otherwise stated): Vitamin A: 500,000 I.E./kg; Vitamin D3: 50,000 I.E./kg; Vitamin E:2500; Vitamin K3 (as Menadione): 1000; Vitamin B1: 5000; Vitamin B2:5000; Vitamin B6: 5000; Vitamin B12: 5; Nicotinic acid: 25000; Pantothenic acid: 10000; Folic acid: 1000; Biotin: 250; Choline chloride: 100000; Inositol: 25000; Vitamin C: 20125.

[2)] Mineral premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg mix): Calcium: 122,160; Phosphorus: 83,670; Magnesium: 14,960; Sodium: 18,180; Potassium: 210,250; Sulfur: 15,460; Chlorine: 29,720; Iron: 1220; Manganese: 1000; Zinc: 1630; Copper: 155; Iodine: 120.

Table 3.2.2 Proximate composition of experimental diets diets containing different levels of JKM and fed to Nile tilapia for 8 weeks.

Diet	Dry Matter % FM	Crude Protein % DM	Crude Lipid % DM	Ash % DM	Gross Energy (kJ / g DM)
J50	88.3	43.5	10.9	10.9	17.5
J75	91.5	43.1	10.2	11.2	18.2
J100	89.0	42.4	9.7	10.6	17.7
BM	91.7	41.3	10.6	9.5	17.8
Control	89.7	43.2	10.8	11.0	19.0

Values represent results of biochemical analysis of diets.

FM = Fresh matter; DM = Dry matter

3.2.3 Results

There were no mortalities within any treatment during the experiment. Palatability of the control diet and all JKM-feeds were good and all given feed was consumed. Water quality parameters (ammonia, nitrite, nitrate, pH) were measured on a weekly basis, while water temperature was monitored daily (Table 3.2.3). Water quality parameters were within the acceptable range for tilapia growth (Redner and Stickney, 1979).

Growth parameters

The fish fed the fishmeal based control diet grew faster than all other treatments (Figure 3.2.1). However, due to large standard deviations, this was not significant for some parameters (Table 3.2.4).

Of the JKM-based diets, diet J50 and J75 showed best growth parameters. SGR, FCR, MGR and PER of these treatments were not significantly different to the fish meal control. Final weights within different treatments of JKM-based diets were not significantly different independent of the replacement level of JKM. LPV and EPV were higher in control fish than diet BM and J100, however not significantly different from diet J50 or J75.

Table 3.2.3 Water parameters measured during the experimental duration.

Week	pH	O_2	NH_4^+	NO_2^-	NO_3^-
1	6.72	7.57	0.031	0.052	945.8
2	6.61	7.68	0.029	0.046	897.8
3	6.60	8.79	0.033	0.047	815.4
4	6.74	8.51	0.055	0.062	-
5	6.65	8.11	0.033	0.059	901.3
6	6.74	8.20	0.027	0.05	882.4
7	6.65	8.71	0.033	0.046	881.2
8	6.51	8.76	0.02	0.069	879.0

Except for pH, values given in mg / l).

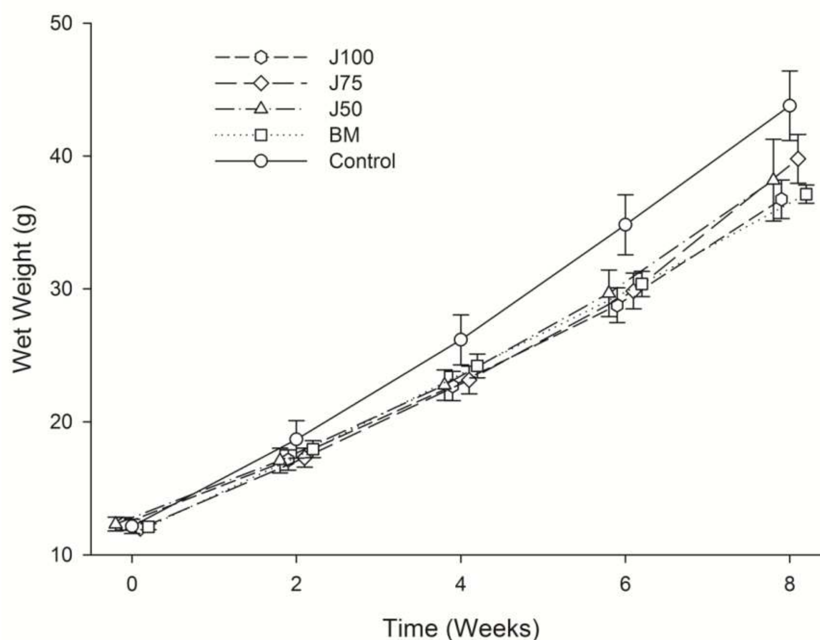


Figure 3.2.1 Body mass development of tilapia (*Oreochromis niloticus*) fed experimental diets based on *Jatropha curcas* kernel meal for 8 weeks.

Development of FCR over time

Figure 3.2.2 shows the differences of FCR-values of experimental fish in four separate 2-week units over the course of the 8-week experiment. After the first two weeks of the experiment, FCRs of diet BM, J50, J75 and were equal to the control diet, while FCR of diet J100 was significantly worse. Over the course of the experiment, FCRs of diets J50, J75, J100 and BM increased up to week six, at which they were all significantly different from the control. This changed for the last two weeks of the experiment where FCR of diets J100 and J50 were equal to the control, whereas diet J75 showed significantly better values than the control.

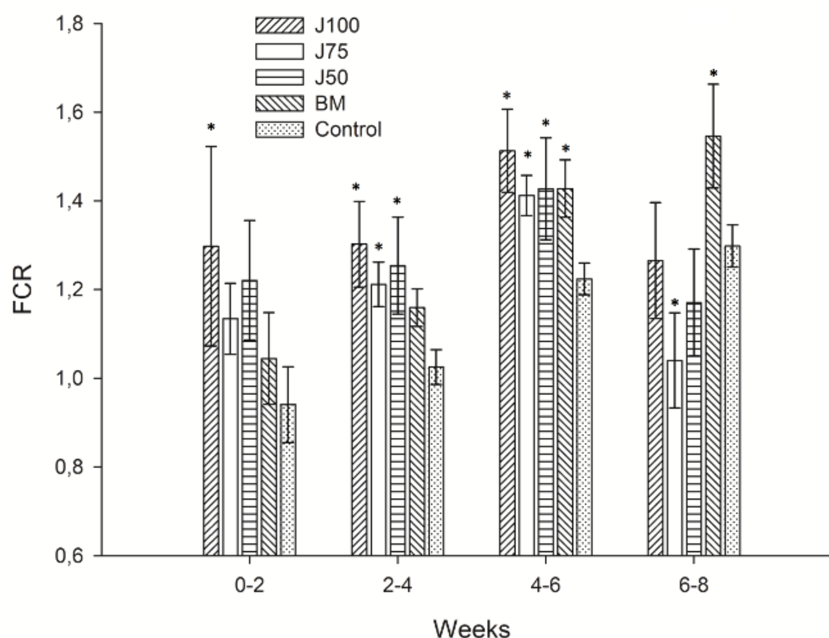


Figure 3.2.2 Development of feed conversion ratio of tilapia (*Oreochromis niloticus*) fed experimental diets based on *Jatropha curcas* kernel meal over the course of the experiment.
*Values differ significantly from the control.

Body composition

Whole body composition of experimental fish is shown in Table 3.2.5. There were no significant differences in dry matter of the fish. Body crude protein was significantly higher in diet J50 than in all other diets. Body lipid content was significantly higher in control fish than in all other treatments and diet J100 and initial fish had significantly lower lipid content than the other treatments. Body ash content of final fish was significantly lower than initial fish. Within final fish, the control diet had lowest ash content, while diets J50, J75 and BM had a similar content. Fish fed diet J100 contained significantly higher ash content than all other treatments.

Table 3.2.4 Growth parameters and nutrient retention of Nile tilapia fed different levels of JKM for 8 weeks.

Treatment	BMG	SGR	FCR	MGR	PER	PPV	LPV	EPV
J50	26.8 ±3.19 [b]	2.06 ±0.18 [a,b]	1.20 ±0.12 [a,b,c]	9.13 ±0.76 [a]	1.94 ±0.20 [a,b]	32.1 ±1.97	38.5 ±3.06 [a,b,c]	24.3 ±1.93 [a,b,c]
J75	27.8 ±1.71 [a,b]	2.14 ±0.07 [a,b]	1.13 ±0.06 [a,b]	9.47 ±0.32 [a,b]	2.05 ±0.10 [a,b]	31.9 ±1.36	44.1 ±6.32 [a,b]	24.9 ±1.63 [a,b]
J100	24.4 ±1.68 [b]	1.95 ±0.12 [b]	1.28 ±0.08 [b,c]	8.64 ±0.48 [b]	1.85 ±0.12 [b]	29.6 ±2.25	32.7 ±5.48 [c]	21.5 ±2.00 [c]
BM	25.0 ±0.73 [b]	2.00 ±0.05 [b]	1.29 ±0.04 [c]	8.84 ±0.19 [b]	1.89 ±0.06 [b]	28.7 ±1.00	34.9 ±4.09 [b,c]	22.4 ±1.22 [b,c]
Control	31.6 ±2.11 [a]	2.29 ±0.04 [a]	1.08 ±0.02 [a]	10.16 ±0.24 [a]	2.15 ±0.05 [a]	32.4 ±2.13	50.1 ±5.28 [a]	26.8 ±1.62 [a]

Values are mean of 5 replicates ± SD. Values that do not share a common superscript differ significantly from one another ($p \leq 0.05$).

BMG, body mass gain (g); SGR specific growth rate (% / day); FCR, feed conversation ratio; MGR, metabolic growth rate (g x kg$^{-0.8}$ day^{-1}); PER, protein efficiency ratio; PPV, protein productive value (%) and LPV, lipid productive value (%) of fish fed *Jatropha curcas*-based experimental diets for 8 weeks.

Table 3.2.5 Proximate composition and gross energy of whole body of Nile tilapia fed different levels of JKM for 8 weeks.

Treatment	Dry Matter		Crude Protein		Crude Lipid		Ash		Gross Energy	
Initial Fish	23.2	±1.76	61.9	±2.75[a,b]	16.6	±1.78[b]	19.9	±1.20[a]	17.0	±0.40[d]
J50	24.0	±0.44	66.6	±1.17[a]	19.2	±1.08[a,b]	16.7	±0.35[b,c]	19.6	±0.21[b,c]
J75	24.3	±0.61	62.4	±0.51[b]	19.4	±1.71[a,b]	16.7	±0.40[b,c]	19.6	±0.53[b,c]
J100	24.2	±0.63	63.9	±1.87[a,b]	16.4	±1.16[b]	17.9	±0.31[b]	18.9	±0.20[c]
BM	23.5	±0.43	63.7	±1.62[a,b]	19.0	±1.12[b]	16.7	±0.91[b,c]	20.1	±0.30[b]
Control	24.1	±0.77	61.8	±1.36[b]	22.0	±1.06[a]	15.9	±0.44[c]	21.1	±0.27[a]

Protein, lipid and ash values based on % dry weight. Gross energy in kJ / g dry weight.
Values are mean of 5 replicates ± SD. Values that do not share a common superscript differ significantly from one another ($p \leq 0.05$).

3.2.4 Discussion

Previous research has already dealt with JKM as a potential replacement for fish meal in tilapia and carp (Kumar et al., 2010a, 2010b, 2011b). Our data shows that growth of fish fed the control diet was significantly faster than all other diets. This is in contrast to previous results, where up to 62.5% of fish meal could be replaced by JKM without any observed difference to the control (Kumar et al., 2010a; Akinleye et al., 2010). However, these authors used JKM from *Jatropha platyphylla*, which is a non-toxic species of the same genus cultivated only in Central America, predominantly Mexico. There might be differences in the amount and type of anti-nutritional factors between these two plants or the detoxification process may have left remainders of phorbol esters slightly affecting growth and leading to the differences in fish performance between these trials.

Despite lower growth at 50% inclusion level of JKM, it would be wrong to dismiss it as an inadequate protein source. For once, growth and FCR of fish fed JKM-based diets was only slightly less than control diets. Secondly, there was no significant difference in feed uptake or growth of fish fed diets with 50% fishmeal replacement and 100% fishmeal replacement. This documents that JKM has the potential to fully

replace fish meal with no adverse effects on palatability and only slight reduction in growth.

The improvement of FCR-values of JKM-based diets in week 6-8 showed an adaptation process of these diets over time. It can therefore be assumed that this adaptation is due to changes in digestive physiology of fish leading to differences in nutrient utilization and therefore FCR. Differences in digestive enzyme activity between tilapia fed a fishmeal- or plant-based diet have been observed before (Sklan et al., 2004). We assume that in our experiment an enzymatic adaptation process of experimental fish towards the JKM-based diets occurred after six weeks into the experiment leading to significantly improved feed conversion during this last period. A feeding trial designed for a longer period than eight weeks is needed to elucidate whether in the long term this would have led to similar growth rates as the fishmeal based control.

The fact that there is no gradual decline in growth rate with increasing JKM content, but almost constant growth within all replacement levels is unusual. Our data suggests that a critical threshold in a growth limiting factor might have been reached at the 50% inclusion level. This might be a limiting amino acid or an anti-nutritional factor of JKM. One possibility is that one of the anti-nutritional factors, e.g. phytate, reaches a critical level at 50% JKM replacement level in the diet already. JKM has a high percentage of the anti-nutritional factor phytate (ca. 9%, Makkar et al., 2009), which has been shown to reduce protein digestibility in rainbow trout (Vielma et al., 2004) and tilapia (Kumar et al., 2010). Phytase was added to our diets, but previous work has shown that this may not completely erase phytate in the diet (Denstadli et al., 2007). The remaining phytate may have been enough to adversely affect mineral and protein absorption (Spinelli et al., 1983, Kumar et al., 2011). On the other hand, Riche et al. (2002, 2004) showed that phytate does not impair nitrogen digestibility

or retention in tilapia fed soybean meal based diets. More research is required in this field to find out on the true impact of phytate on growth of tilapia.

Final fish fed the control diet had significantly higher body lipid percentage than all other treatments. Similarly, the lipid productive value was also significantly higher. These findings are contrary to previous results where replacing fishmeal with plant-based feedstuffs many times resulted in higher lipid accumulation in the fish body. This is thought to be due to low protein quality and therefore diets, of which the available protein/energy ratio is too low. Consequently, dietary energy cannot be spent on protein production and is deposited as fat (Kaushik et al., 2004). Wee and Shu (1989) also observed increasing body fat content in tilapia fed full fat soy bean meal based diets over a fish meal control diet. On the other hand, Garduno-Lugo et al. (2008) replaced fish meal with peanut meal and found decreasing body fat levels with increasing inclusion of peanut meal. El-Saidy et al. (2003) found no changes in body fat content of tilapia when fed a diet containing a mixture of various plant-based protein sources. It may be assumed that fish in our experiment were not amino acid limited and therefore retrieved all available energy in growth leaving no additional energy to build fat reserves. It may be speculated that a higher energy containing diet could have further improved growth up to the level of the control diet.

So far, there are no plant-based feedstuffs, which can completely replace fish meal in diets for tilapia without decline in growth parameters. In comparison to previous results, it can therefore be concluded that JKM is a promising plant protein source in the future. We have shown that it can serve as a sole protein source with only slight reductions in growth compared to a fishmeal based diet when amino acids are added according to the requirement of tilapia. Whether JKM will become an alternative to fish meal in commercial diets will depend on its price and the price of the detoxification process (Makkar and Becker, 2009). Further research to lower the feed cost, for example by releasing phosphorus from phytate through phytase application or

combining other plant based feedstuffs to minimize artificial amino acid supplementation is required. It may be said that on an absolute scale, full replacement of fishmeal by JKM was successful and further trials need to confirm whether comparable growth rates can be observed in a commercial pond scenario.

Acknowledgements

The project was partly supported by funds of the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program. Further funding was granted by the University of Stirling, Stirling, UK. Thanks to the staff of the Thuenen Institute in Ahrensburg for support in fish rearing and laboratory matters.

3.2.5 References

Akinleye, A. O., Kumar, V.; Makkar, H. P. S.; Angulo-Escalante, M. A.; Becker, K., 2011: *Jatropha platyphylla* kernel meal as feed ingredient for Nile tilapia (*Oreochromis niloticus* L.): Growth, nutrient utilization and blood parameters. Journal of Animal Physiology and Animal Nutrition 96, 119-129.

Boisen, S.; Bech-Andersen, S.; Eggum, B. O., 1987: A critical view on the conversion factor 6.25 from total nitrogen to protein. Acta Agriculturae Scandinavica 37, 299-304.

Committee on the Nutrient Requirements of Fish and Shrimp of the National Research Council, 2011: *Nutrient Requirements of Fish and Shrimp*. The National Academic Press.

Denstadli, V.; Storebakken, T.; Svihus, B.; Skrede, A., 2007: A comparison of online phytase pre-treatment of vegetable feed ingredients and phytase coating in diets for Atlantic salmon (*Salmo salar* L.) reared in cold water. Aquaculture 269, 414-426.

El-Saidy, D. M. S. D.; Gaber, M. M. A., 2002: Complete replacement of fish meal by soybean meal with dietary l-lysine supplementation for Nile tilapia *Oreochromis niloticus* (L.) fingerlings. Journal of the World Aquaculture Society 33, 297–306.

El-Saidy, D. M. S. D.; Gaber, M. M. A., 2003: Replacement of fish meal with a mixture of different plant protein sources in juvenile Nile tilapia, *Oreochromis niloticus* (L.) diets. Aquaculture Research 34, 1119-1127.

Garduño-Lugo, M.; Olvera-Novoa, M. Á., 2008: Potential of the use of peanut (*Arachis hypogaea*) leaf meal as a partial replacement for fish meal in diets for Nile tilapia (*Oreochromis niloticus* L.). Aquaculture Research 39, 1299-1306.

Jauncey, K., 1982: The effects of varying dietary protein level on the growth, food conversion, protein utilization and body composition of juvenile tilapias (*Sarotherodon mossambicus*). Aquaculture *27*, 43-54.

Kaushik, S. J.; Covès, D.; Dutto, G.; Blanc, D., 2004: Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. Aquaculture 230, 391–404.

Köprücü, K.; Özdemir, Y., 2005: Apparent digestibility of selected feed ingredients for Nile tilapia (*Oreochromis niloticus*). Aquaculture 250, 308-316.

Kumar, V.; Akinleye, O.; Makkar, H. P. S.; Angulo-Escalante, M.; Becker, K., 2010a: Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing *Jatropha platyphylla* kernel meal as a protein source. Journal of Animal Physiology and Animal Nutrition 96, 37-46.

Kumar, V.; Makkar, H. P. S.; Becker, K., 2010b: Dietary inclusion of detoxified *Jatropha curcas* kernel meal: Effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L. Fish Physiology and Biochemistry 36, 1159-1170.

Kumar, V.; Makkar, H. P. S.; Devappa, R. K.; Becker, K., 2011a: Isolation of phytate from *Jatropha curcas* kernel meal and effects of isolated phytate on growth, digestive physiology and metabolic changes in Nile tilapia (*Oreochromis niloticus* L.). Food and Chemical Toxicology 49, 2144-2156.

Kumar, V.; Makkar, H. P. S.; Becker, K., 2011b: Detoxified *Jatropha curcas* kernel meal as a dietary protein source: Growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings. Aquaculture Nutrition 17, 313-326.

Lin, S.; Luo, L., 2011: Effects of different levels of soybean meal inclusion in replacement for fish meal on growth, digestive enzymes and transaminase activities in practical diets for juvenile tilapia, *O. niloticus*×*O. aureus*. Animal Feed Science and Technology 168, 80–87.

Makkar, H. P. S.; Becker, K., 2009: *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added co-products. European Journal of Lipid Science and Technology 111, 773-787.

Makkar, H.; Becker, K., 2011: Detoxification of *Jatropha curcas* meal for feeding to farm animal species and fish. *United States Patent Application Publication*.

NRC 2011: *Nutrient Requirements of Fish and Shrimp*. National Research Council, Washington DC. The National Academic Press. 376 pp.

Redner, B. D.; Stickney, R. R., 1979: Acclimation to ammonia by *Tilapia aurea*. Transaction of the American Fisheries Society 108, 383–388.

Riche, M.; Trottier, N. L.; Ku, P. K.; Garling, D. L., 2002: Apparent digestibility of crude protein and apparent availability of individual amino acids in tilapia (*Oreochromis niloticus*) fed phytase pretreated soybean meal diets. Fish Physiology and Biochemistry 25, 181–194.

Riche, M.; Garling, D. L., 2004: Effect of phytic acid on growth and nitrogen retention in tilapia, *Oreochromis niloticus* L. Aquaculture Nutrition 10, 389-400.

Sklan, D.; Prag, T.; Lupatsch, I., 2004: Structure and function of the small intestine of the tilapia *Oreochromis niloticus* x *Oreochromis aureus*. Aquaculture Research 35, 350–357.

Spinelli, J.; Houle, C.; Wekell, J., 1983: The effect of phytates on the growth of rainbow trout (*Salmo gairdneri*) fed purified diets containing varying quantities of calcium and magnesium. Aquaculture 30, 71-83.

Vielma, J.; Ruohonen, K.; Gabaudan, J.; Vogel, K., 2004: Top-spraying soybean meal-based diets with phytase improves protein and mineral digestibilities but not lysine utilization in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture Research 35, 955-964.

Wee, K. L.; Shu, S.-W., 1989: The nutritive value of boiled full-fat soybean in pelleted feed for Nile tilapia. Aquaculture 81, 303–314.

Yousif, O. M.; Alhadhrami, G. A.; Pessaraklib, M., 1994: Evaluation of dehydrated alfalfa and salt bush (*Atriplex*) leaves in diets for tilapia (*Oreochromis aureus*). Aquaculture 126, 341-347.

3.3 Influence of a sodium formate / formic acid mixture on growth of juvenile common carp (*Cyprinus carpio*) fed different fishmeal replacement levels of detoxified *Jatropha curcas* kernel meal in practical, mixed diets.

December 2013 – April 2014

Carsten Krome[1,2], Fabian Schuele[2], Kim Jauncey[1], Ulfert Focken[2]

[1)] Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, UK

[2)] Thuenen Institute of Fisheries Ecology, Wulfsdorfer Weg 204, 22926 Ahrensburg, Germany

Abstract

An experiment was conducted to test the suitability of detoxified *Jatropha curcas* kernel meal (JKM) on its own and in combination with a dietary organic acid / salt blend (NDF) on practical diets for common carp (*Cyprinus carpio*). Eight feeds were prepared, replacing 25% of fishmeal protein with JKM at 0% (Control), 30% (30%), 70% (70%) and 100% (100%). All diets were produced again including 0.5% of NDF (0% + NDF; 30% + NDF; 70% + NDF and 100% + NDF). A total of 200 carp (five fish per replicate, five replicates per treatment, initial weight: 8.65 ± 0.21 g) were placed in 45 L aquaria connected to a recirculation system and fed experimental diets at five times their maintenance ration for eight weeks. Statistical analysis (multifactorial ANOVA followed by Dunnett test) showed significantly higher final weights (FW) and lower feed conversion ratios (FCR) for treatments 30% + NDF (FW: 26.2 ± 2.60 g, p = 0.013; FCR: 1.49 ± 0.05, p = 0.029) and 100% + NDF (FW: 25.9 ± 1.03 g, p = 0.026; FCR: 1.50 ± 0.07, p = 0.040) compared to the control (FW: 22.6 ± 1.36 g and FCR: 1.75 ± 0.09). No interacting effects of JKM and NDF were observed.

Body moisture content was significantly lower (p = 0.03) and body lipid (p = 0.01) and energy content (p = 0.00) higher in JKM based diets. Dietary NDF supplementation significantly increased body moisture (p = 0.03) and lowered body lipid (p = 0.11) and energy content (p = 0.05) compared to non-supplemented diets. This was

supported by a significant decreasing effect of NDF on blood plasma cholesterol levels ($p = 0.02$).

In conclusion, the present trial shows JKM to be a suitable protein source to replace fishmeal in diets for carp. Moreover, dietary inclusion of 0.5% of an organic acid / salt blend increases growth performance and influences lipid metabolism of carp. Further research should focus on long-term, field testing of JKM as a protein source with organic acid / salt blend inclusion.

3.3.1 Introduction

Organic acids and their salts have been used as growth promoting agents and as a replacement for antibiotic growth promoters (AGPs). AGPs were banned by the European Union in 1999 due to dangers associated with the risk of resistance in pathogenic bacteria (Casewell, 2003). The functional mechanisms of organic acids have not been fully resolved. Organic acids are thought to express antibiotic activity by passively diffusing through the cell wall of bacteria, dissociating and decreasing the pH inside the cytoplasm thereby altering the homeostatic balance of the cell, ultimately leading to cell death (Defoirdt et al., 2009). This is supposed to benefit the microbial flora in the digestive system leading to better growth. Alternative growth promoting functions are proposed to be changing gut pH to a favorable degree for proteolytic enzymes (Knarreborg et al., 2002) or using the acids as an alternative energy source thereby potentially increasing the protein sparing effect (Partanen & Mroz, 1999). As reviewed by Lueckstädt et al. (2008), salts of organic acids increased growth rate in Arctic charr (1% sodium lactate) or tilapia (0.5% potassium diformate). Improved growth was also shown for abalone fed diets containing either a blend of organic acids or their salts compared to diets without supplementation (Goosen et al., 2011).

To this date JKM has not been investigated as a protein source in practical diets involving a variety of plant-based feedstuffs in carp. The present trial examines the effects of JKM replacement of fishmeal in common carp (*Cyprinus carpio*) on growth parameters, body composition and blood plasma composition and whether an organic acid / salt blend can positively contribute to utilization of JKM.

3.3.2 Material and Methods

Diet Formulation

Detoxified JKM was provided by JatroSolutions GmbH, Stuttgart, Germany. An additional moist heat treatment step was conducted in an autoclave (30 min, 121°C at 66% moisture) to reduce trypsin inhibitor from 3.5 mg / g to 0.47 mg / g (analysis of trypsin inhibitor conducted by the Institute of Animal Nutrition at the University of Hohenheim, Hohenheim, Germany). After moist heat treatment, the meal was freeze-dried and ground with a laboratory grinder. Nutrient and amino acid analysis (Table 3.3.1) of the meal was conducted by LUFA-ITL GmbH in Kiel, Germany. Eight isonitrogenous and isoenergetic diets (gross energy content: 20 kJ / g) were produced containing 35% protein and 12% lipid (50% fish oil, 50% sunflower oil). Of the 35% total protein, 25% percent was provided by fishmeal (Diet Control). 30%, 70% and 100% of the fishmeal protein fraction was replaced by JKM in the respective treatment diets (Diet “30%”, “70%”, “100%”). All diets were also produced with a 0.5% content of a mixture of sodium formate and formic acid sold under the brand name “NDF” (supplied by ADDCON GmbH, Bonn, Germany, supplement concentration applied as recommended by the company). These diets were named Control + NDF, 30% + NDF, 70% + NDF and 100% + NDF. Vitamins and minerals for all diets were supplied in the form of a premix. Essential amino acids were added to meet the requirements of carp (NRC, 2011). The composition of the experimental diet is shown in Table 3.3.3. Dietary ingredients were mixed with a standard kitchen blend-

er. The organic acid / salt blend was added to the ingredient mixture and evenly distributed before 40% water (w/v) was added. The mix was passed through a meat mincer to form noodles, which were then crumbled to palatable size and air-dried at 45° or 48 hours.

Experimental animals and setup

Cyprinus carpio of the mirror carp strain were hatched and reared with standard commercial carp feed to the initial experimental weight of 8.65 ± 0.21 g at the Thuenen Institute of Fisheries Ecology, Ahrensburg, Germany. Four weeks before start of the trial, fish were graded and the faster growing batch was used for a different experiment. Of the slower growing batch, a total of 200 experimental fish (five replicates for each of the 8 experimental diets, each replicate containing 5 fish) were distributed into a total of forty 45 L-aquarium tanks connected to a recirculation system. Water temperature was 26°C, with 1.5 L / min/tank flow rate. Fish were acclimatized to the system for one week. The experimental duration was 8 weeks. Water quality parameters (ammonia, nitrite, nitrate, pH and O_2, Redner & Stickney, 1979) were measured on a weekly basis. Faeces were collected daily throughout the last two weeks of the experiment by stripping the hindgut of the fish and returning them to the tank immediately.

As a parameter for feed quantity, the metabolic maintenance ration of the fish was calculated (see 2.1.3).

Pre- and post experimental procedures, chemical analyses as well as performance calculations and and equations were conducted as outlined in Chapter 2.

Statistical analyses

Shapiro-Wilk test was used to test for normal distribution of the values. Two-factorial ANOVA ($p \leq 0.05$) was used to analyze treatments (JKM replacement level, organic acid / salt supplementation and the interaction of these two). In addition, all treatments were compared to the control diet though a Dunnett test and deemed signifi-

cantly different at $p \leq 0.05$. Percentages were arcsine transformed before analysis. Statistics were conducted with Statistica 8 software. Values are expressed as mean ± standard deviation.

3.3.3 Results

Growth

Feed palatability was good and all rations were consumed quickly at all times. There were no mortalities during the experiment. The growth parameters are shown in Table 3.3.4. Fish body mass increased between 158% (100%) and 202% (30% + NDF) over the experimental period.

Table 3.3.1 Nutrient (% dry weight) and amino acid (g / 100g protein) composition of JKM.

Nutrient	Amount
Dry matter	94.7
Crude protein	53.1
Crude lipid	< 1.0
Crude ash	13.2
Crude fiber	22.3
-ADF	28.4
-NDF	42.6
-ADL	14.0
Amino acids	
Lysine	3.16
Methionine	1.45
Cystine	1.39
Aspartic acid	8.93
Threonine	3.46
Serine	4.51
Glutamic acid	15.19
Proline	4.12
Glycine	4.25
Alanine	4.57
Valine	5.03
Isoleucine	4.51
Leucine	6.80
Tyrosine	2.39
Phenylalanine	4.43
Histidine	2.49
Arginine	10.46
Tryptophane	1.07

Analysis conducted by LUFA-ITL GmbH, Kiel, Germany.

1) Acid detergent fibre

2) Neutral detergent fibre

3) Acid detergent lignin

Table 3.3.2 Nutrient (%) and essential amino acid (EAA, g / 100g protein) composition of feedstuffs used in the experimental diets.

Nutrient	Fishmeal[1)]	SPC[2)]	Rice bran[2)]	Wheat meal[2)]
Dry Matter	92.8	93.3	91.0	88.9
Crude Protein	69.8	68.2	15.9	12.3
Crude Lipid	10.5	0.2	22.5	2.1
Crude Ash	14.1	0.0	12.8	2.2
Crude Fiber	0.0	4.5	12.2	11.1
NFE	5.6	27.1	36.7	72.3
EAA				
Arginine	5.41	7.29	7.69	7.35
Histidine	2.71	2.48	2.62	3.33
Isoleucine	3.49	4.62	3.38	4.36
Leucine	6.38	7.73	7.08	7.86
Lysine	6.27	6.18	4.38	4.96
Phenylalanine	3.34	5.15	4.31	4.70
Methionine	2.45	1.27	2.00	1.71
Threonine	3.53	3.88	3.69	3.93
Tryptophan	0.75	1.32	1.08	2.14
Valine	4.49	4.81	5.23	5.90

[1)] EAAs measured by Landesanstalt fuer Landwirtschaftliche Chemie, Hohenheim, Germany
[2)] EAA compositions taken from NRC, 2011

The treatment diets in general showed equal or improved growth parameters compared the control diet. ANOVA revealed a significant influence of JKM and NDF supplementation on final weight and feed conversion ratio. No interaction between these factors could be observed. Dunnett test for differences to the control treatment showed treatment 30% + NDF and 100% + NDF to have significantly improved final weight ($p = 0.013$ and $p = 0.026$, respectively) and feed conversion ($p = 0.029$ and $p = 0.04$, respectively). Treatment 30% + NDF also had improved specific growth rate and protein efficiency ratio compared to the control.

Table 3.3.3 Composition of experimental diets containing different replacement levels of fishmeal with JKM with and without NDF.

Ingredient	Control	Control + NDF	30%	30% + NDF	70%	70% + NDF	100%	100% + NDF
Fish meal	13.5	13.5	9.5	9.5	4.1	4.1	0.0	0.0
Wheat meal	20.9	20.9	20.9	20.9	20.9	20.9	20.9	20.9
SPC	32.1	32.1	32.1	32.1	32.1	32.1	32.1	32.1
Rice bran	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0
JKM	0.0	0.0	4.9	4.9	11.3	11.3	16.2	16.2
Sun flower oil	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
Fish oil	4.7	4.7	5.1	5.1	5.6	5.6	6.0	6.0
Cellulose	5.3	4.8	3.9	3.4	2.2	1.7	0.8	0.3
Vitamin premix[1)]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Mineral premix[2)]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
NaH_2PO_4	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Lysine[3)]	0.24	0.24	0.35	0.35	0.51	0.51	0.62	0.62
Methionine	0.14	0.14	0.18	0.18	0.23	0.23	0.26	0.26
Threonine	0.30	0.30	0.31	0.31	0.33	0.33	0.34	0.34
NDF[4)]	0.00	0.50	0.00	0.50	0.00	0.50	0.00	0.50
Yttrium oxide	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Phytase (U / kg)[5)]	1000	1000	1000	1000	1000	1000	1000	1000
Proximate analysis								
Dry matter	93.8	92.0	94.2	94.7	93.3	94.3	93.5	94.8
Crude protein	35.9	35.2	36.2	36.2	35.9	35.8	35.7	36.2
Lipid	12.2	12.8	12.0	12.2	12.5	12.3	12.7	12.2
Ash	9.24	9.24	9.24	9.23	9.27	9.24	9.27	9.24
P (g / kg)	13.7	13.7	13.4	14.0	12.9	12.7	12.6	12.1
Ca (g / kg)	9.4	9.7	8.3	8.3	6.6	6.5	5.1	4.9
Mg (g / kg)	3.3	3.2	3.8	3.9	4.2	4.0	4.6	4.4
Fe (mg / kg)	146	140	237	233	295	296	358	359
Cu (mg / kg)	9.0	8.2	11.0	11.1	13.4	11.8	14.2	13.2
Zn (mg / kg)	70.9	69.3	73.3	74.4	70.5	69.1	73.4	71.4
Gross energy (kJ / g)	20.1	19.6	20.3	20.3	20.0	20.1	19.9	20.1
NDF[6)]	0.0	0.41	0.0	0.66	0.0	0.53	0.0	0.53

SPC: soy protein concentrate. Values of ingredients based on % fresh weight, values of proximate analysis based on % dry weight.

[1)] Vitamin premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg, unless otherwise stated): Vitamin A: 500,000 I.E./kg; Vitamin D3: 50,000 I.E./kg; Vitamin E:2500; Vitamin K3 (as Menadione): 1000; Vitamin B1: 5000; Vitamin B2:5000; Vitamin B6: 5000; Vitamin B12: 5; Nicotinic acid: 25000; Pantothenic acid: 10000; Folic acid: 1000; Biotin: 250; Choline chloride: 100000; Inositol: 25000; Vitamin C: 20125.

[2)] Mineral premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg mix): Calcium: 122,160; Magnesium: 14,960; Sodium: 18,180; Potassium: 210,250; Sulfur: 15,460; Chlorine: 29,720; Iron: 1220; Manganese: 1000; Zinc: 1630; Copper: 155; Iodine: 120.

[3] supplied as lysine monohydrochloride

[4] NDF (sodium formate / formic acid mix) supplied by ADDCON GmbH, Bonn, Germany

[5] Ronozyme P (L), DSM

[6] Measured by Addcon Europe GmbH, Bitterfeld-Wolfen, Germany

Body composition

Body composition is shown in Table 3.3.5. ANOVA revealed a significant difference in impact of JKM replacement level and NDF supplementation on moisture, lipid and energy content. High JKM replacement levels showed lower moisture and higher lipid and energy contents than lower replacement levels. The opposite was true for NDF supplementation, however, only moisture and energy was significant, but not lipid content. There were no differences in body protein and ash content. Mineral composition showed significant differences in relation to JKM supplementation only for iron and in relation to NDF only for zinc. Higher JKM supplementation increased body iron content, while NDF supplementation lowered body zinc content. There were no differences of single treatments compared to the control. No interactions between different JKM supplementation levels and NDF supplementation could be observed as well as any differences of treatments to the control.

Digestibility and nutrient retention

There was no significant difference in dry matter or protein digestibility between treatments (Table 3.3.6), however, a tendency for higher values in the Control treatment could be observed. Nutrient retention analysis showed highly significant improvements for nitrogen, phosphorus

Table 3.3.4 Growth parameters of common carp fed experimental diets containing different levels of JKM with and without NDF for 8 weeks.

Parameter	Diet								ANOVA		
	Control	**Control + NDF**	**30%**	**30% + NDF**	**70%**	**70% + NDF**	**100%**	**100% + NDF**	**JKM**	**NDF**	**JKM * NDF**
FW	22.6 ± 1.36	23.4 ± 0.64	24.7 ± 2.31	26.2 ± 2.60 [a]	24.0 ± 0.71	24.7 ± 2.86	22.2 ± 1.36	25.9 ± 1.03 [a]	0.03	0.00	0.19
SGR	1.73 ± 0.11	1.78 ± 0.04	1.87 ± 0.21	1.97 ± 0.16 [a]	1.82 ± 0.07	1.88 ± 0.20	1.69 ± 0.11	1.93 ± 0.06	0.06	0.01	0.37
FCR	1.75 ± 0.09	1.66 ± 0.05	1.57 ± 0.22	1.49 ± 0.15 [a]	1.62 ± 0.06	1.59 ± 0.21	1.79 ± 0.13	1.50 ± 0.07 [a]	0.05	0.01	0.18
PER	1.59 ± 0.09	1.71 ± 0.05	1.79 ± 0.25	1.87 ± 0.19 [a]	1.72 ± 0.07	1.79 ± 0.24	1.57 ± 0.12	1.84 ± 0.08	0.10	0.01	0.42

FW: Final Weight; SGR: Specific growth rate (% / day); FCR: Feed conversion ratio; PER: Protein efficiency ratio. Values with the superscript “a” are significantly different from treatment “Control” (Dunnett test, $p \leq 0.05$).

Two-way ANOVA with impact of JKM, NDF and interaction of JKM and NDF on parameters, ($p \leq 0.05$).

Table 3.3.5 Body composition of common carp before and after fed different levels of JKM with and without NDF for 8 weeks.

Parameter	Diet									ANOVA		
	Initial Fish	**Control**	**Control + NDF**	**30%**	**30% + NDF**	**70%**	**70% + NDF**	**100%**	**100% + NDF**	**JKM**	**NDF**	**JKM * NDF**
Moisture	77.5 ± 0.67	78.1 ± 0.16	79.2 ± 0.49	78.1 ± 1.40	79.0 ± 0.87	77.4 ± 0.11	78.2 ± 1.12	77.5 ± 1.02	77.5 ± 0.96	0.03	0.03	0.61
Crude protein	14.6 ± 0.33	14.1 ± 0.43	13.7 ± 0.27	13.7 ± 0.73	13.7 ± 0.68	14.2 ± 0.10	13.9 ± 0.36	13.9 ± 0.26	14.3 ± 0.27	0.20	0.64	0.44
Crude lipid	3.82 ± 0.38	4.69 ± 0.27	4.04 ± 0.50	5.09 ± 0.71	4.57 ± 0.12	5.52 ± 0.24	4.91 ± 1.10	5.40 ± 0.92	5.62 ± 0.48	0.01	0.11	0.48
Ash	3.35 ± 0.18	2.43 ± 0.10	2.53 ± 0.19	2.32 ± 0.14	2.36 ± 0.22	2.39 ± 0.06	2.37 ± 0.06	2.31 ± 0.04	2.34 ± 0.06	0.08	0.46	0.84
P (g / kg)	4.50 ± 0.24	4.40 ± 0.14	4.50 ± 0.15	4.32 ± 0.27	4.27 ± 0.53	4.48 ± 0.24	4.23 ± 0.21	4.36 ± 0.16	4.28 ± 0.20	0.71	0.47	0.73
Ca (g / kg)	5.65 ± 0.29	5.77 ± 0.38	6.15 ± 0.39	5.82 ± 0.48	5.73 ± 0.97	6.09 ± 0.39	5.57 ± 0.34	5.84 ± 0.55	5.58 ± 0.36	0.83	0.54	0.49
Mg (g / kg)	0.29 ± 0.01	0.27 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	0.25 ± 0.03	0.27 ± 0.01	0.25 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	0.78	0.13	0.90
Fe (mg / kg)	16.9 ± 0.64	11.0 ± 0.77	11.0 ± 0.69	10.9 ± 0.97	10.4 ± 1.43	10.9 ± 0.56	11.1 ± 1.68	12.8 ± 1.30	12.0 ± 0.35	0.02	0.53	0.81
Cu (mg / kg)	1.53 ± 0.05	1.41 ± 0.09	1.36 ± 0.16	1.65 ± 0.43	1.23 ± 0.14	1.66 ± 0.14	1.61 ± 0.25	1.60 ± 0.25	1.64 ± 0.53	0.30	0.29	0.43
Zn (mg / kg)	136 ± 3.65	72.2 ± 3.98	67.4 ± 5.16	70.2 ± 5.62	62.9 ± 10.8	72.4 ± 4.47	67.3 ± 4.44	76.3 ± 4.65	64.6 ± 3.22	0.54	0.00	0.58
Gross energy (kJ / kg)	4.89 ± 0.27	4.95 ± 0.01	4.70 ± 0.22	5.13 ± 0.07	4.80 ± 0.21	5.36 ± 0.10	5.04 ± 0.42	5.34 ± 0.35	5.44 ± 0.18	0.00	0.05	0.36

Two-way ANOVA with impact of JKM, NDF and interaction of JKM and NDF on parameters, ($p \leq 0.05$). Values in % unless otherwise stated and based on fresh weight. No differences to the control were detected (Dunnett test).

and magnesium, but not for calcium, in NDF supplemented diets (Table 3.3.7). No differences in nitrogen, lipid or phosphorus contents were observed for JKM supplementation, however, significant differences were observed for energy, calcium and magnesium retention. Compared to the control, calcium retention was significantly higher for all treatments containing 70% or more JKM, while magnesium retention was lower for these treatments.

Blood plasma parameters showed a significant effect of NDF inclusion on total cholesterol (Table 3.3.8). Diets containing NDF had lower cholesterol levels than non-NDF supplemented diets ($p = 0.02$). There was a tendency for NDF to also raise total protein content of plasma, however, this was not significant ($p = 0.09$). The same was true for the influence of JKM on albumin content ($p = 0.10$). No differences were observed for plasma glucose, triglycerides or inorganic phosphate.

Table 3.3.6 Dry matter and protein digestibility of common carp fed different levels of JKM with and without NDF for 8 weeks.

Treatment	Dry matter	Protein
Control	61.6 ± 6.51	85.9 ± 3.64
Control + NDF	56.9 ± 15.1	79.4 ± 11.4
30%	51.9 ± 7.48	77.2 ± 8.20
30% + NDF	49.1 ± 9.67	79.3 ± 6.38
70%	48.1 ± 12.97	82.4 ± 4.85
70% + NDF	48.8 ± 5.47	80.1 ± 5.37
100%	48.9 ± 8.37	78.8 ± 3.89
100% + NDF	50.7 ± 5.97	75.0 ± 2.51
ANOVA[1)]		
JKM	0.14	0.40
NDF	0.74	0.89
JKM * NDF	0.93	0.36

Values in %. Values are means ± SD of 5 replicates. Faeces are pooled samples from five fish.
Two-way ANOVA with impact of JKM, NDF and interaction of JKM and NDF on parameters, ($p \leq 0.05$).
No differences to the control were detected (Dunnett test).

Table 3.3.7 Nutrient and mineral retention of common carp fed different contents of JKM with and without NDF for 8 weeks.

Parameter	Diets								ANOVA		
	Control	**Control + NDF**	**30%**	**30% + NDF**	**70%**	**70% + NDF**	**100%**	**100% + NDF**	**JKM**	**NDF**	**JKM * NDF**
Nitrogen	22.4 ± 1.61	23.8 ± 0.38	24.2 ± 2.99	25.8 ± 1.69	22.4 ± 1.69	24.5 ± 2.93	21.3 ± 1.58	25.2 ± 2.20	0.25	0.01	0.65
Lipid	23.6 ± 2.49	25.1 ± 11.9	29.2 ± 6.56	26.4 ± 4.10	31.5 ± 1.08	28.9 ± 5.82	28.7 ± 8.02	34.5 ± 3.26	0.16	0.83	0.50
Energy	11.2 ± 1.28	10.7 ± 1.66	11.9 ± 1.29	13.4 ± 1.95	13.8 ± 0.36	13.9 ± 0.38	12.7 ± 2.78	15.7 ± 0.83 [a]	0.01	0.12	0.24
Phophorus	18.0 ± 0.36	20.5 ± 0.87	20.0 ± 2.62	20.6 ± 1.78	19.5 ± 1.35	21.3 ± 3.78	18.1 ± 1.41	21.2 ± 1.26	0.64	0.01	0.56
Calcium	36.5 ± 1.56	41.4 ± 3.26	44.8 ± 6.13	48.0 ± 7.15	54.0 ± 4.71 [a]	56.0 ± 7.03 [a]	62.2 ± 9.33 [a]	70.0 ± 5.81 [a]	0.00	0.08	0.79
Magnesium	4.45 ± 0.30	5.01 ± 0.21	4.26 ± 0.60	4.29 ± 0.26	3.49 ± 0.16 [a]	3.90 ± 0.61	2.90 ± 0.30 [a]	3.42 ± 0.21 [a]	0.00	0.01	0.50

Values with the superscript “a” are significantly different from treatment “Control” (Dunnett test, $p \leq 0.05$).

Two-way ANOVA with impact of JKM, NDF and interaction of JKM and NDF on parameters, ($p \leq 0.05$).

Table 3.3.8 Blood plasma parameters of common carp fed different contents of JKM with and without NDF for 8 weeks.

Parameter	Diets								ANOVA		
	Control	**Control + NDF**	**30%**	**30% + NDF**	**70%**	**70% + NDF**	**100%**	**100% + NDF**	**JKM**	**NDF**	**JKM * NDF**
TP	3.05 ± 0.19	2.92 ± 0.24	3.14 ± 0.15	3.0 ± 0.16	3.30 ± 0.22	3.04 ± 0.47	3.14 ± 0.23	3.10 ± 0.19	0.46	0.09	0.82
ALB	0.73 ± 0.10	0.65 ± 0.17	0.70 ± 0.10	0.70 ± 0.07	0.76 ± 0.09	0.88 ± 0.21	0.82 ± 0.15	0.75 ± 0.06	0.10	0.86	0.37
GLU	74.3 ± 32.9	81.0 ± 23.4	89.6 ± 27.9	74.6 ± 29.2	94.0 ± 35.2	73.8 ± 14.8	92.2 ± 27.8	81.5 ± 18.3	0.91	0.28	0.73
TCHO	94.8 ± 10.6	92.5 ± 9.26	103.0 ± 9.25	89.4 ± 11.2	98.8 ± 7.85	90.0 ± 14.0	96.8 ± 8.26	90.0 ± 8.29	0.93	0.02	0.68
TG	198.5 ± 12.6	187.4 ± 3.58	191.6 ± 11.5	189.8 ± 16.2	187.4 ± 19.5	185.0 ± 17.7	193.6 ± 16.0	192.8 ± 13.2	0.71	0.40	0.87
IP	9.74 ± 3.26	9.90 ± 2.97	10.8 ± 2.32	10.0 ± 2.23	11.5 ± 2.16	11.8 ± 3.14	12.2 ± 2.28	10.1 ± 1.79	0.43	0.47	0.71

TP: Total protein (g / dl); ALB: Albumin (g / dl); GLU: Glucose (mg / dl); TCHO: Total cholesterol (mg / dl); TG: Total triglycerides (mg / dl); IP: Inorganic phosphate (mg / dl). Two-way ANOVA with impact of JKM, NDF and interaction of JKM and NDF on parameters, ($p \leq 0.05$).

3.3.4 Discussion

JKM as a dietary protein source in practical diets for common carp

According to Tacon & Metian (2008) commercial carp diets contained an average of 5% fishmeal in the year 2007 with the remaining protein coming from plant based sources. It is therefore of more practical interest to investigate the performance of JKM in mixed-plant based diets compared to trials that supplement JKM in diets where fish meal is the only protein source (Kumar et al., 2010a, 2011a, 2012; Krome et al., 2014). The present trial attempts to replicate a practical diet for carp as closely as possible at the same time leaving enough fish meal in the diet to assess its replacement with JKM.

As highlighted in the introduction, no trials so far with JKM as a protein source have succeeded in replacing fishmeal at the maximum level without compromising growth compared to the control diet (Kumar et al., 2010a, 2011a, 2012; Harter et al., 2011; Krome et al., 2014). The present results show that for carp, fishmeal can be replaced by JKM at 100% in practical diets without worsening of growth parameters, actually even showing a positive effect on final weight and feed conversion when replaced at 30% or 70%. Kumar et al. (2011a) compared a 50% and 75% replacement level of fish meal with JKM in basic diets containing fishmeal and wheat meal as the only protein sources for carp. They found fish fed the 75% replacement level to grow significantly slower than the fishmeal control diet and hypothesized that anti-nutritional factors, such as phytate, in the meal might be the reason. The same authors isolated *Jatropha* protein by isoelectric precipitation and found fish fed diets containing 75% *Jatropha* protein to grow equally fast compared to control diets (Kumar et al., 2011b). In both these experiments, growth parameters were better compared to the present trial. This may have to do with the higher dietary protein content applied by these authors (38%), more frequent feeding (five instead of three

daily rations) or the fact that fish in the present trial had to be selected from the slower growing part of the batch (see Material & Methods). Markovic et al. (2012) fed a diet containing 15% fishmeal to juvenile carp with the remaining protein coming from several different plant feedstuffs. In the present study, control fishmeal concentration was of similar content (13.5%) with the remaining protein coming from soy protein concentrate, rice bran and wheat meal. Feed conversion ratios in both these trials were similar. Hence, the observed feed conversion ratios in the present trial are within the normal limits of practical diets for carp.

In the present trial, dry matter and protein digestibility did not reflect growth parameters. Though not significant, a tendency for higher digestibilities could be observed for the control treatment, despite equal growth. Kumar et al. (2011a) documented significantly worse dry matter digestibility despite equal body mass gain in carp fed 50% of protein through JKM compared to the control. Crude fibre content of JKM in the present trial was very high (21.3%, Table 3.3.1). Diets all contained the same crude fibre content through the addition of cellulose. However, fibre analysis of JKM shows substantial proportions of fibre coming from hemicelluloses and lignin. A modest cellulase activity has been observed for *Cyprinus carpio* (Lindsay & Harris, 1980), but it is unlikely that hemicelluloses and lignin can be digested. Slightly reduced dry matter digestibility in the present data is therefore assumed to originate from higher concentrations of these components in JKM based diets. The non-significant differences in protein digestibility could also be connected to differences in fibre composition. JKM analysis shows neutral detergent fibre to be 42.6% of total fibre with the remainder being neutrally soluble. Soluble and non-soluble fibre change digesta viscosity (Leenhouwers et al., 2006), which may change gut transit time of feed and influence protein assimilation (Krogdahl et al., 2005).

Body protein and ash content was similar for all dietary treatments. Body moisture, energy and lipid content were affected by the JKM supplementation level with in-

creasing substitution resulting in increasing body lipid content. Since all diets were isonitrogenous and isoenergetic, this may be a sign of a more unbalanced amino acid composition or availability of those diets. Cheng et al. (2003) reported lower body lipid levels in rainbow trout fed a largely fishmeal based control diet than for a diet containing more plant-based feedstuffs. This effect was less evident when 1% crystalline lysine was supplemented. It is likely that despite fulfilling the latest amino acid requirements in the present trial (NRC, 2011), some amino acids may have been less available to the fish therefore leading to higher lipid deposition in the body. Body iron content was higher in high JKM supplements than in low ones. This is certainly due to higher iron concentration in these diets. The principle role of iron in the organism is as a component of haemoglobin and myoglobin, but it also functions as a co-factor of a variety of enzymes. Iron is assimilated by the mucosa through specific transport proteins. Due to its low solubility, excess iron is difficult to excrete and deposited in cells of the digestive system (NRC, 2011). The higher body iron concentrations observed in the present trial may derive from excessive deposition in these cells.

Blood plasma parameters were measured to obtain evidence on potential physiological impacts of the experimental diets. Pradhan et al. (2014) established reference values for wild gibel carp *Labeo rohita*. Compared to these values, the present trial showed 10% lower total protein, lower 25% lower cholesterol, but 30% higher plasma glucose values. However, nutritional differences between wild and cultured fish are obvious.

Several authors observed a decrease in plasma cholesterol when plant-based contents of feeds were increased and this is attributed to withdrawal of cholesterol-rich fishmeal (Kaushik et al., 2004; Kumar et al., 2011c). In the present trial this was not observed for increasing levels of JKM. Initial fishmeal inclusion was already low and

this is supported by control cholesterol values being low on an absolute scale (Pradhan et al., 2014).

The present trial showed a correlation of body lipid content to JKM content (Table 3.3.5). Therefore higher triglyceride values could have been expected, but this was not observed (Kumar et al., 2010b). Fish were not fed the last day of the experiment when blood was withdrawn, which could have influenced plasma triglyceride values as these are considered a short-term indicator of lipid metabolism (Kumar et al., 2010b). Inorganic phosphate content in the present study did not show significant differences and was similar to Nwanna et al. (2007) feeding *Cyprinus carpio* with a diet of comparable composition.

Impact of an organic acid / salt mix (NDF) on development of common carp fed JKM-based diets

The addition of NDF significantly improved final weight, specific growth rate, feed conversion ratio, protein efficiency ratio and protein productive value compared to non-supplemented diets. Treatments 30% + NDF and 100% + NDF showed significantly improved growth parameters compared to the control. The results demonstrate that NDF may positively contribute to a mixed plant based diet, especially when containing JKM. In an experiment by Baruah et al. (2007), it could be shown that 3% citric acid inclusion in a mixed diet containing 5% fishmeal for the cyprinid rohu (*Labeo rohita*) increased specific growth rate, protein efficiency ratio and protein productive value compared to a non-supplemented diet. Interestingly, a negative correlation was observed between citric acid addition and protein content of the diet: Weight gain in fish fed a citric-acid supplemented 35% protein diet was less pronounced than fish fed a citric-acid supplemented diet containing 25% protein, while weight gain of fish fed a 25% protein and a 35% protein diet without citric acid supplementation was equal. Results by Baruah et al. (2007) were confirmed in a trial with almost identical design for common carp (Khajepour et al., 2012a).

There was no influence of NDF on dry matter or protein digestibility by experimental fish in the present trial. This is similar to results by Baruah et al. (2007). The increment in growth parameters was, however, reflected in improved nitrogen, phosphorus and magnesium retention. With equal dry matter and nitrogen digestibilities but better mineral retention, the positive growth effect of NDF must therefore be rooted in improved mineral digestibilities or a process beyond the digestive system. Unfortunately, phosphorus digestibility could not be measured in this trial as the amount of faeces collected was not sufficient. Improved phosphorus, calcium and magnesium digestibilities through organic acid addition have already been observed by Vielma & Lall (1997) for rainbow trout. Likewise, phosphorus bone mineralization was improved through 3% citric acid in rohu (Baruah et al., 2005). Possible reasons for the effect of organic acids on mineral digestibility could be the release of minerals from the phytate complex in plant-based diets. Ng et al. (2009) observed a decrease of microbial colony forming units (CFU / g) from 1.81 to 0.67 CFU / g in faeces and 1.81 to 0.68 CFU / g in gut of tilapia fed 0.2% of potassium diformate in the diet compared to a control thereby clearly identifying *in vivo* antimicrobial action of the substance. Microbes may utilize and catabolize nutrients in the digestive tract, which thus cannot be used by the fish.

Analysis of body composition documents significant differences of NDF inclusion on moisture and energy content. Lipid content was not significantly different ($p = 0.11$), however, a tendency towards lower body lipid values through NDF inclusion can be assumed. This is interesting as it indicates an influence of organic acids / salts on the energy metabolism of carp and is similar to a study conducted on Beluga (*Huso huso*), in which body lipid was significantly decreased through addition of 3% citric acid to diets containing different concentrations of soy bean meal (Khajepour & Hosseini, 2012b).

There was a significant lowering impact of NDF on plasma cholesterol ($p = 0.02$). This also supports the evidence that organic acids and / or their salts could interfere with lipid metabolism as seen in Baruah et al. (2007) who observed significantly lower body lipid content in *Labeo rohita* juveniles when including 3% citric acid in the diet.

Conclusion

The present trial shows the suitability of JKM as a protein source in aquaculture diets for carp. This 8 week laboratory scale experiment showed no adverse effects in growth even at a 100% replacement level. Moreover, addition of 0.5% organic acid / salt blend significantly enhanced growth and nitrogen and mineral retention as well as influenced lipid metabolism. Longer term trials with JKM as a protein source including organic acids / salts are required to confirm these preliminary findings.

3.3.5 References

Baruah, K., Pal, A. K., Sahu, N. P., Jain, K. K., Mukherjee, S. C., & Debnath, D. (2005). Dietary protein level, microbial phytase, citric acid and their interactions on bone mineralization of *Labeo rohita* (Hamilton) juveniles. *Aquaculture Research*, *36*(8), 803–812.

Baruah, K., Sahu, N. P., Pal, A. K., Jain, K. K., Debnath, D., & Mukherjee, S. C. (2007). Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. *Aquaculture Research*, *38*(2), 109–120.

Casewell, M., Friis, C., Marco, E., McMullin, P., & Phillips, I. (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *The Journal of Antimicrobial Chemotherapy*, *52*(2), 159–161.

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*(4), 271–277.

Cho, C., & Slinger, S. (1979). Apparent digestibility measurement in feedstuffs for rainbow trout. In *Finfish Nutrition and Fishfeed Technology* (Vol. 2, pp. 239–247).

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., & Bossier, P. (2009). Short-chain fatty acids and poly-beta-hydroxyalkanoates: (New) Biocontrol agents for a sustainable animal production. *Biotechnology Advances*, *27*(6), 680–685.

Goosen, N. J., Görgens, J. F., De Wet, L. F., & Chenia, H. (2011). Organic acids as potential growth promoters in the South African abalone *Haliotis midae*. *Aquaculture*, *321*(3-4), 245–251.

Harter, T., Buhrke, F., Kumar, V., Focken, U., Makkar, H. P. S., & Becker, K. (2011). Substitution of fish meal by *Jatropha curcas* kernel meal: Effects on growth performance and body composition of white leg shrimp (*Litopenaeus vannamei*). *Aquaculture Nutrition*, *17*(5), 542–548.

Khajepour, F., Hosseini, S. A., & Imanpour, M. R. (2012a). Dietary crude protein, citric acid and microbial phytase and their interacts to influence growth performance, muscle proximate composition and hematocrite of common carp, *Cyprinus carpio* L, juveniles. *World Journal of Zoology*, *7*(2), 118–122.

Khajepour, F., & Hosseini, S. A. (2012b). Citric acid improves growth performance and phosphorus digestibility in Beluga (*Huso huso*) fed diets where soybean meal partly replaced fish meal. *Animal Feed Science and Technology*, *171*(1), 68–73.

Kaushik, S. J., Covès, D., Dutto, G., & Blanc, D. (2004). Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. *Aquaculture*, *230*(1-4), 391–404.

Knarreborg, A., Miquel, N., Granli, T., & Jensen, B. B. (2002). Establishment and application of an *in vitro* methodology to study the effects of organic acids on coliform and lactic acid bacteria in the proximal part of the gastrointestinal tract of piglets. *Animal Feed Science and Technology*, *99*(1-4), 131–140.

Krogdahl, A., Hemre, G.-I., & Mommsen, T. P. (2005). Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. *Aquaculture Nutrition*, *11*(2), 103–122.

Krome, C., Jauncey, K., Fedderke, S., & Focken, U. (2014). Effect of replacing different levels of dietary fishmeal with *Jatropha curcas* kernel meal on the development of Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758). *Journal of Applied Ichthyology*, *30*(3), 507–512.

Kumar, V., Makkar, H. P. S., & Becker, K. (2010a). Dietary inclusion of detoxified *Jatropha curcas* kernel meal: effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L. *Fish Physiology and Biochemistry*, *36*(4), 1159–70.

Kumar, V., Makkar, H. P. S., Amselgruber, W., & Becker, K. (2010b). Physiological, haematological and histopathological responses in common carp (*Cyprinus carpio* L.) fingerlings fed with differently detoxified *Jatropha curcas* kernel meal. *Food and Chemical Toxicology, 48*(8-9), 2063–2072.

Kumar, V., Makkar, H. P. S., & Becker, K. (2011a). Detoxified *Jatropha curcas* kernel meal as a dietary protein source: growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings. *Aquaculture Nutrition*, *17*(3), 313–326.

Kumar, V., Makkar, H. P. S., & Becker, K. (2011b). Evaluations of the nutritional value of *Jatropha curcas* protein isolate in common carp (*Cyprinus carpio* L.). *Journal of Animal Physiology and Animal Nutrition*, *96*(6), 1030–1043.

Kumar, V., Makkar, H. P. S., & Becker, K. (2011c). Nutritional, physiological and haematological responses in rainbow trout (*Oncorhynchus mykiss*) juveniles fed detoxified *Jatropha curcas* kernel meal. *Aquaculture Nutrition*, *17*(4), 451–467.

Kumar, V., Akinleye, A. O., Makkar, H. P. S., Angulo-Escalante, M. A., & Becker, K. (2012). Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing *Jatropha platyphylla* kernel meal as a protein source. *Journal of Animal Physiology and Animal Nutrition*, *96*(1), 37–46.

Leenhouwers, J. I., Verreth, J. A. J., & Schrama, J. W. (2006). Digesta viscosity, nutrient digestibility and organ weights in African catfish (*Clarias gariepinus*) fed diets supplemented with different levels of a soluble non-starch polysaccharide. *Aquaculture Nutrition*, *12*, 111–116.

Lindsay, G. J. H., & Harris, J. E. (1980). Carboxymethylcellulase activity in the digestive tracts of fish. *Journal of Fish Biology*, *16*(3), 219–233.

Lueckstaedt, C. (2008). The use of acidifiers in fish nutrition. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *3*(044), 1–8.

Marković, Z., Poleksić, V., Lakić, N., Živić, I., Dulić, Z., Stanković, M., … Sørensen, M. (2012). Evaluation of growth and histology of liver and intestine in juvenile carp (*Cyprinus carpio*, L.) fed extruded diets with or without fish meal. *Turkish Journal of Fisheries and Aquatic Sciences*, *12*, 301–308.

Ng, W.-K., Koh, C.-B., Sudesh, K., & Siti-Zahrah, A. (2009). Effects of dietary organic acids on growth, nutrient digestibility and gut microflora of red hybrid tilapia, *Oreochromis* sp., and subsequent survival during a challenge test with *Streptococcus agalactiae*. *Aquaculture Research*, *40*(13), 1490–1500.

NRC, National Research Council (2011). *Nutrient Requirements of Fish and Shrimp*. The National Academic Press, Washington.

Nwanna, L. C., & Schwarz, F. J. (2007). Effect of supplemental phytase on growth, phosphorus digestibility and bone mineralization of common carp (*Cyprinus carpio* L). *Aquaculture Research*, *38*(10), 1037–1044.

Partanen, K. H., & Mroz, Z. (1999). Organic acids for performance enhancement in pig diets. *Nutrition Research Reviews*, *12*(1), 117–45.

Pradhan, S. C., Patra, a. K., & Pal, a. (2014). Hematological and plasma chemistry of Indian major carp, Labeo rohita (Hamilton, 1822). *Journal of Applied Ichthyology*, *30*(1), 48–54.

Redner, B. D., & Stickney, R. R. (1979). Acclimation to Ammonia by Tilapia aurea. *Transaction of the American Fisheries Society*, *108*(4), 383–388.

Storebakken, T., Shearer, K. D., & Roem, A. J. (2000). Growth , uptake and retention of nitrogen and phosphorus , and absorption of other minerals in Atlantic salmon *Salmo salar* fed diets with fish meal and soy-protein concentrate as the main sources of protein. *Aquaculture Nutrition*, *6*(2), 103–108.

Tacon, A. G. J., & Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture*, *285*(1-4), 146–158.

Vielma, J., & Lall, S. P. (1997). Dietary formic acid enhances apparent digestibility of minerals in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Nutrition*, *3*(4), 265–268.

3.4 *Jatropha curcas* kernel meal as a replacement for fish meal in practical Nile tilapia (*Oreochromis niloticus*, Linaeus, 1758) feeds

July 2012 – November 2012

Carsten Krome[1,2], Kim Jauncey[1], Sybille Fedderke[2], Ulfert Focken[2]

[1)] Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, UK

[2)] Thuenen Institute of Fisheries Ecology, Wulfsdorfer Weg 204, 22926 Ahrensburg, Germany

Abstract

Jatropha curcas is an upcoming oil-seed plant with increasing cultivation area each year throughout the tropics. After de-oiling the seeds, a protein-rich meal (JKM) is left behind with a similar amino acid composition and content compared to fishmeal. To test JKM as an alternative protein source, a mixed diet was formulated in which 25% of the total dietary protein was derived from fishmeal and the rest from soybean meal, rice bran and wheat meal (Control). Three further isonitrogenous and isoenergetic diets replacing 30% (30%), 70% (70%) or 100% (100%), respectively, of the fishmeal with JKM were produced and all diets were fed to juvenile tilapia (*Oreochromis niloticus*) for 8 weeks. There were no significant differences in growth parameters between all treatments containing JKM, however, regression analysis revealed a significant negative correlation of JKM content to final weight and specific growth rate. The 70% and 100% replacement levels showed higher body lipid and significantly lower body ash content than the control and the 30% diet. There was a significant negative correlation of JKM content and dry matter digestibility, but no differences in protein digestibility between the treatments. In summary, JKM is a promising alternative protein source in aquaculture diets for tilapia, though slower growth and higher body fat of fish fed JKM in this experiment suggest the need for further research to improve the nutritional value of JKM.

3.4.1 Introduction

Research on JKM as a protein source in fish diets has resulted in promising first results for culture fish such as common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*) and rainbow trout (*Oncorhynchus mykiss*) (Kumar et al. 2010, 2011a, 2011b; Akinleye et al. 2012; Kumar et al. 2012; Krome et al. 2014) and white leg shrimp (*Penaeus vannamei*) (Harter et al. 2011). To date, experiments have been conducted with JKM as the sole protein source replacing fishmeal as the sole control protein source. In tilapia farming, fishmeal levels in feeds have already been significantly reduced (Hardy, 2010) and it is therefore of greater value to investigate replacement of fishmeal by JKM in feeds with mixed protein sources. The present experiment investigated the extent to which it is possible to replace fishmeal protein with JKM in a practical diet containing 25% fishmeal protein and the remaining protein originating from soybean meal, rice bran and wheat meal.

3.4.2 Material and Methods

Experimental Diets

Four isonitrogenous (32% crude protein), isoenergetic diets were formulated with detoxified JKM replacing, 30%, 70% and 100% of the 25% fishmeal protein in the control diet. Amino acids were added to ensure feeds met requirements (NRC 2011). The nutrient composition of detoxified JKM is shown in Table 3.4.1. Pre-experimental *in vitro* trials had shown 2000 U / kg phytase (Ronozyme, DSM) to have a maximum effect on JKM-phytate hydrolysis, which is why this concentration was chosen for all diets. Yttrium oxide was added as a digestibility marker.

Table 3.4.1 Nutrient (g / kg) and amino acid (g / kg protein) composition of detoxified JKM in comparison to fishmeal and nutrient and essential amino acid composition of other applied feedstuffs.

Nutrients	JKM[2]	FM[3]	SBM[4]	Rice bran[4]	Wheat meal[4]	ΔFM[5]
Dry Matter	947.2	928.4	900.0	910.0	888.7	
Crude Protein	598.9	698.0	535.0	154.0	126.0	
Crude Lipid	1.2	105.0	33.0	209.0	21.0	
Crude Ash	139.3	141.0	81.1	127.5	21.6	
Crude Fiber	79.0	0.0	70.0	122.0	111.4	
NFE[1]	128.8	0.0	282.9	387.5	720.0	
Essential Amino Acids						
Arginine	104.7	54.1	74.2	54.1	73.5	50.6
Histidine	34.6	27.1	26.8	18.5	33.3	7.5
Isoleucine	40.5	34.9	53.6	32.5	43.6	5.5
Leucine	72.7	63.8	78.4	64.3	78.6	8.9
Lysine	34.2	62.7	46.2	34.4	49.6	-28.5
Phenylalanine	46.1	33.4	55.7	35.7	47.0	12.7
Methionine	20.0	24.5	14.4	13.4	16.2	-4.5
Threonine	37.2	35.3	41.2	28.7	39.3	2.0
Tryptophane	11.4	7.5	14.4	13.4	21.4	3.8
Valine	46.2	44.9	55.7	41.4	59.0	1.3
Non-essential amino acids						
Alanine	49.1	61.6				-12.5
Asparginine	114.7	86.7				28.0
Cystine	3.8	6.2				-2.3
Glycin	52.6	85.7				-33.1
Glutamine	187.2	113.8				73.4
Proline	53.8	52.9				0.9
Serine	51.1	36.5				14.6
Tyrosine	31.4	21.2				10.2

[1)] NFE calculated as: 100 – (CP + CL + CA + CF + H_2O)

[2)] Data determined by Rodehutscord and colleagues at the University of Hohenheim, Germany

[3)] Data from Akinleye et al. (2011)

[4)] Data from NRC (2011)

[5)] JKM - FM

Table 3.4.2 Composition and proximate analysis of diets containing different levels of JKM fed to Nile tilapia for 8 weeks.

Ingredient (g kg^{-1})	Control	30%	70%	100%
Fish meal	123.0	86.1	36.9	0.0
Wheat meal	227.2	216.0	204.5	194.2
Soybean meal	365.0	365.0	365.0	365.0
Rice bran	200.0	200.0	200.0	200.0
JKM	0.0	43.0	98.0	140.0
Fish oil	38.0	42.0	46.0	50.0
Vitamin premix[a]	20.0	20.0	20.0	20.0
Mineral premix[b]	20.0	20.0	20.0	20.0
Lysine	1.2	2.2	3.5	4.5
Methionine	2.2	2.4	2.7	2.9
Threonine	1.3	1.3	1.4	1.4
Histidine	1.8	1.8	1.7	1.7
Yttrium oxide	0.3	0.3	0.3	0.3
Phytase (U / kg)	2000	2000	2000	2000
Proximate analysis				
Dry matter	949.3	949.1	959.1	956.0
Crude protein	317.1	314.2	308.6	313.8
Lipid	100.7	104.3	101.1	101.7
Ash	91.9	92.2	91.4	89.1
Gross energy (kJ / g)	18.8	19.0	18.8	18.9
Protein : energy (mg / kJ)	16.9	16.5	16.4	16.6

[a] Vitamin premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg premix, unless otherwise stated): Vitamin A: 500,000 I.U./kg; Vitamin D3: 50,000 I.U./kg; Vitamin E: 2,500; Vitamin K3 (as Menadione): 1,000; Vitamin B1: 5,000; Vitamin B2: 5,000; Vitamin B6: 5,000; Vitamin B12: 5; Nicotinic acid: 25,000; Pantothenic acid: 10,000; Folic acid: 1,000; Biotin: 250; Choline chloride: 100,000; Inositol: 25,000; Vitamin C: 20,125.

[b] Mineral premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg premix): Calcium: 122,160; Phosphorus: 83,670; Magnesium: 14,960; Sodium: 18,180; Potassium: 210,250; Sulfur: 15,460; Chlorine: 29,720; Iron: 1,220; Manganese: 1,000; Zinc: 1,630; Copper: 155; Iodine: 120.

The composition of experimental diets is shown in Table 3.4.2. Dietary ingredients were weighed and mixed in a kitchen blender. Distilled water was added to form a paste which was then passed through a meat mincer and the resulting strands

crumbled to pieces of palatable size. These were dried at 45°C for 48 hours in a well-ventilated, heated chamber.

Experimental Design

16 tanks with 40 L net volume (4 diets and n = 4 replicates) of four all-male tilapia (initial weight: 4.94 ± 0.29 g, obtained from Kirschauer Aquakulturen, Schirgiswalde-Kirschau, Germany) were set up in a randomized pattern and connected to a recirculation system. Aerated water at 1.5 L/min at 26°C was supplied to each tank. Water quality parameters (DO level, pH, nitrite, ammonia) were measured weekly and values were below critical levels for tilapia. Photoperiod was 14:10 hours light: dark regime. Feed was supplied at five times the basal requirement, by hand, divided into three feeds per day (0800, 1300, and 1700) for 8 weeks.

As a parameter for feed quantity, the metabolic maintenance ration of the fish was calculated (see 2.1.3).

Pre- and post experimental procedures, chemical analyses as well as performance calculations and and equations were conducted as outlined in Chapter 2.

Statistics

One-way analysis of variance (ANOVA) and linear regression were used to analyze treatments. The significance level was set to 5% ($p < 0.05$). Tukey HSD test was applied as post-hoc test and percentages were arcsine transformed before analysis. Statistics were conducted with Statistica 8.0 software. Values are expressed as means ± standard deviation.

3.4.3 Results

Fish grew well over the experimental period, feed palatability was good and all feed was consumed at all times. Due to there being only four fish per aquarium, combat behaviour was observed leading to mortalities between 6.25 and 31.25% dependent on treatment (Table 3.4.3). When a fish died, it was weighed and feeding rate of the

respective fish tank was adjusted immediately. There was no connection between mortality and the extent of fishmeal replacement of the different treatments (p = 0.73).

Table 3.4.3 Survival of experimental fish fed diets containing different levels of JKM for 8 weeks.

Control	30%	70%	100%	R^2	P
75.0 ± 28.9	93.8 ± 12.5	68.8 ± 12.5	81.3 ± 23.9	0.01	0.73

Values in %. R^2: Mortality values regressed to the dietary level of JKM.

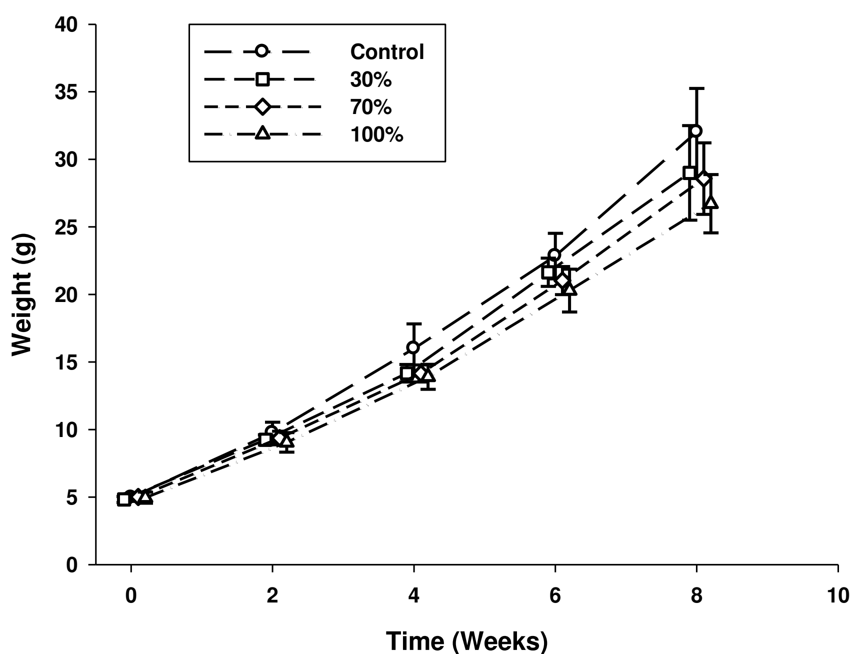


Figure 3.4.1 Body weight development of Nile tilapia fed different levels of JKM for 8 weeks.
Error bars represent standard deviation of the mean.

Figure 3.4.1 shows the weight development of fish over the experimental period. Body mass gain of the control group was highest and there was a significant negative correlation between specific growth rate of the control diet and treatments 30%, 70% and 100% (R^2 = 0.29; p = 0.031, Table 3.4.5). Feed conversion ratio for the

control diet was lower, but no significant difference or correlation could be observed ($R^2 = 0.08$, $p = 0.28$). The same counts for nutrient productive values (Table 3.4.5).

Body composition of experimental fish is documented in Table 3.4.4. Crude lipid content of fish fed all JKM-based diets was higher, with the differences of diet 70% being significant compared to the control diet. In contrast, ash values of fish fed 70% and 100% were significantly lower than 30% and the control diet. There were significant correlations between diets for lipid and ash content, respectively ($R^2 = 0.46$, $p = 0.00$; $R^2 = 0.81$, $p = 0.00$). In agreement with the higher lipid content, body energy contents were also higher in treatments 70% and 100%. No differences or correlations of body dry weight or protein content were observed.

There was a significant negative correlation of dry matter digestibility of the control diet to the treatment diets (Table 3.4.6). Protein digestibility was similar in all treatments. Energy digestibility was significantly higher in the control diet than in diet 30%, but there were no differences of these treatments to both other ones.

Table 3.4.4 Body composition of experimental fish fed different levels of JKM for 8 weeks.

Treatment	**Initial Fish**	**Control**	**30%**	**70%**	**100%**	**R^2**	**P**
Moisture	76.8 ± 1.76	76.3 ± 0.32	76.6 ± 0.92	75.5 ± 1.24	75.9 ± 1.03	0.08	0.32
Crude Protein	13.4 ± 1.01	15.1 ± 0.56	14.5 ± 0.74	14.9 ± 0.62	15.1 ± 0.30	0.01	0.71
Crude Lipid	4.4 ± 0.99	4.5 ± 0.47 ^{a}	4.8 ± 0.34 ^{a,b}	6.0 ± 0.84 ^{b}	5.9 ± 0.95 ^{a,b}	0.46	0.00
Ash	5.0 ± 0.80	3.7 ± 0.14 ^{b}	3.6 ± 0.11 ^{b}	3.0 ± 0.10 ^{a}	2.9 ± 0.07 ^{a}	0.81	0.00
Gross energy (kJ/g)	4.3 ± 0.22	4.7 ± 0.14	4.8 ± 0.22	5.3 ± 0.32	5.2 ± 0.29	0.40	0.01

Values in % fresh weight. Mean values not sharing the same superscript differ significantly from one another ($p \leq 0.05$). R^2: values are regressed to the dietary level of JKM.

Table 3.4.5 Growth performance parameters and nutrient utilization of fish fed different levels of JKM for 8 weeks.

Treatment	Control		30%		70%		100%		R^2	P
IW	4.96	±0.28	4.83	±0.22	5.01	±0.30	4.96	±0.42		
FW	32.0	±3.23	29.0	±3.50	28.6	±2.65	26.7	±2.15	0.33	0.020
SGR	3.32	±0.27	3.19	±0.26	3.10	±0.17	3.01	±0.07	0.29	0.031
FCR	1.00	±0.20	1.02	±0.15	1.03	±0.09	1.10	±0.06	0.08	0.280
PER	3.23	±0.57	3.18	±0.42	3.16	±0.27	2.89	±0.15	0.11	0.222
PPV	49.3	±6.97	46.9	±7.61	45.8	±6.83	44.6	±2.51	0.09	0.265
LPV	45.9	±12.1	47.0	±3.81	59.1	±16.4	55.1	±8.21	0.16	0.127
EPV	26.3	±4.93	25.7	±3.93	27.2	±5.22	25.9	±1.45	0.00	0.970

IW, initial weight (g); FW, final weight; SGR, specific growth rate (%/day); FCR, feed conversation ratio; PER, protein efficiency ratio; PPV, protein productive value (%); LPV, lipid productive value (%); EPV, energy productive value (%). R^2: Respective values are regressed to the dietary level of JKM.

Table 3.4.6 Effect of feeding different levels of JKM on apparent digestibility coefficients of dry matter, protein and energy in Nile tilapia.

Treatment	Control			30%			70%			100%			R^2	P
Dry matter	78.5	±	2.70	75.7	±	0.76	73.6	±	3.62	75.4	±	0.42	0.25	0.05
Protein	90.9	±	2.09	91.1	±	2.82	90.5	±	1.37	89.8	±	0.65	0.08	0.31
Energy	83.2	±	0.58^a	80.9	±	1.34^b	80.7	±	$1.34^{a,b}$	81.1	±	$0.53^{a,b}$	0.25	0.10

Values in %. Mean values not sharing the same superscript differ significantly from one another ($p \leq 0.05$)

3.4.4 Discussion

The significant negative correlation of JKM inclusion in terms of SGR shows that JKM has a negative impact on growth. Previous research on JKM as a protein source was mostly conducted by Kumar et al. (2011a, 2011b, 2012). 62.5% of fishmeal protein was replaced by JKM produced from *Jatropha platyphylla*, a different species endemic to Central America, in diets for tilapia with equal growth rates compared to the control diet and a diet where the same percentage of fishmeal was replaced by soybean meal (Kumar et al. 2012). Similar results were also reported by Akinleye et al. (2012). JKM from *Jatropha curcas* has also successfully replaced

fishmeal in other fish species at levels of 50% and 75% in carp (Kumar et al. 2010; Kumar et al. 2011a) and 50% in rainbow trout (Kumar et al. 2011b). In these studies, slower growth at higher inclusion levels was attributed to anti-nutritional factors present in JKM, especially phytate. Kumar et al. (2011c) have isolated a phytate-rich fraction of JKM and added this fraction to a standard diet resulting in slower growth. Phytate from plant-based feedstuffs is known to bind minerals such as zinc, calcium, iron or phosphorus in the digestive tract lowering their bioavailability in the animal (Maga 1982). However, Sajjadi and Carter (2004) showed for salmon that addition of phytate to the diet had no impact on growth. In a previous study, no difference in protein productive value between diets containing phytase and a control diet without phytase with both diets containing additional phosphate could be observed (Krome et al., in press). Spinelli et al. (1983) observed reduced amino acid availability in diets containing phytic acid. However, Riche et al. (2001), showed that there was reduced amino acid availability of soybean meal based diets in tilapia caused by phytase pre-treatment and attributed this to the removal of phytates. Altogether it is unlikely that the reduced growth observed in the experiments by Kumar et al. (2011a) and in our JKM treatments arose from reduced mineral or amino acid availability caused by phytate. Other anti-nutritional factors, such as oxalate, NSPs or left-over trypsin inhibitor or phorbol esters are more likely to have been the cause (Francis et al. 2001; Makkar and Becker 2009).

Specific growth rates between 3.01% day^{-1} (100%) and 3.32% day^{-1} (Control) are high compared to other experiments with similar sized tilapia of the same species. Our 25% fishmeal control diet is comparable to the 75% plant-protein replacement level of El-Saidy and Gaber (2003), who used a plant protein mix consisting of soybean, cottonseed, sunflower and linseed. The specific growth rate in their experiment (1.5% day^{-1}) was a lot lower and FCR (2.0) a lot higher than in our experiment with water temperatures and starting size of fish being similar to the present study

and feeding to apparent satiation twice daily. A possible reason for this discrepancy may be that the present diets were based on the most recent amino acid requirement data (NRC 2011), which differ from the older ones used by these authors in terms of higher required histidine and lysine content (NRC 1993). This might have led to a more balanced amino acid profile and thus better protein utilisation.

The present study control diet contained a mixture of different plant based protein sources with 25% of the protein coming from fishmeal (inclusion level of 12,3%) and the remainder from soybean, rice bran and wheat flour. Trosvik et al. (2012) used a control diet containing 20% fishmeal, the rest of the protein coming from soy bean and wheat flour. In their treatments, they replaced the remaining fishmeal with additional soybean meal and observed significantly reduced growth with and without addition of crystalline lysine and methionine. In the present experiment, JKM seems to replace fishmeal more effectively than soybean meal in the experiment by Trosvik et al. (2012). JKM has between 10-20% higher protein content than soybean meal dependent on the source (Makkar and Becker, 2009). Therefore less JKM needs to be included in the diet to achieve equal protein contribution compared to soybean meal. Anti-nutritional factors present in both soybean and JKM such as NSP (Kumar et al. 2011a) are therefore relatively less in diets where the fishmeal is replaced by JKM compared to those where fishmeal is replaced by soybean meal which may result in a difference in growth.

Fontainhas-Fernandes et al. (1999) have investigated the replacement of fish meal in tilapia diets with a mixture of isonitrogenous and isoenergetic plant-based feedstuffs. Starting fish weight was similar to the present trial and dietary protein contents were similar. Despite fulfilling tilapia essential amino acid requirements (NRC 1993), fish grew significantly slower even at the 33% replacement level than control fish. In experiments on rainbow trout, *Oncorhynchus mykiss*, Gaye-Siessegger et al.

(2007, 2011) have tested the effect of replacing the full spectrum of non-essential amino-acids as found in fish meal by either the metabolic precursors of the respective AAs (glutamate, aspartate, serine and phenylalanine) or only glutamate in isonitrogenous diets and found a gradual decrease in growth rates with decreased spectrum of AAs. In the present experiment, a large part of non-essential AAs is glutamate (187.2 g / kg protein in JKM vs. 113.8 g / kg in FM, Table 3.4.1) and this may have lead to an adverse effect in growth. Ideally, non-essential amino acids are also taken into account when designing a diet that replaces fishmeal protein with plant based feedstuffs. Feed applied by Fontainhas-Fernandes et al. (1999) may also have had a different non-essential amino acid composition compared to their fishmeal control diet. The non-essential amino acid composition of the lower feedstuffs in our experiment may have been closer to the requirements potentially explaining why they found a significant decline in growth at 33% inclusion level unlike in our experiment, where replacement of 30% showed worse, but not significantly worse body mass gain.

Tacon and Metian (2008) estimated an average 5% inclusion of fish meal in commercial tilapia diets for the year 2007. With this inclusion an average FCR of 1.7 was achieved in commercial systems. The present experiment resulted in an FCR of 1.1 with 0% fishmeal inclusion although the study was not under commercial conditions and was only 8 weeks in duration.

Body composition of experimental fish showed a higher lipid content in diets with high JKM inclusion. This is a typical phenomenon observed in fish fed plant-based diets: due to a suboptimal balance of amino acids provided, growth is inhibited and remaining energy originally supplied for growth is converted into fat tissue (Kaushik et al. 2004). Despite providing amino acids according to the latest standards of the NRC (2011) in the present trial, there may still have been a shortage of a particular

essential amino acid. The diets applied in this trial had protein to energy ratios (mg / kJ) between 16.4 (70%) and 16.9 (control), which is lower than the optimum (Mazid et al. 1979). This may have had led to a more pronounced effect of fat deposition the higher the replacement level of the respective diet.

Dependent on market price of JKM, it might be more economical to dispense with fishmeal entirely despite slightly slower growth. The market price for JKM is currently unknown and difficult to estimate as the key step towards a useful product is the detoxification process to dispose of toxic phorbol esters and trypsin inhibitor. Studies to identify commercially viable large-scale processes to detoxify JKM are under way. Furthermore, availability of press cake from *Jatropha curcas* seeds will depend on the future of this plant as an oilseed crop. This depends on the performance and oil yield of *Jatropha* seeds under commercial plantation conditions in arid or semi-arid areas and it is still under discussion whether *Jatropha curcas* is suitable for the task (Openshaw 2000).

Conclusion

As *Jatropha curcas* is likely to play an increasingly important role as an oil-seed crop, its significance as a feedstuff in diets for aquaculture will also increase. This study shows how detoxified JKM can be used in mixed diets for tilapia demonstrating a gradual decline in SGR with higher JKM inclusions, but still showing good growth rates of fish fed on 100% JKM. Further research on JKM as a feedstuff for tilapia should focus on more realistic and if possible long-term pond trials possibly incorporating different feed additives to minimize the effects of anti-nutritional factors besides phytate.

Acknowledgements

The project was partly supported by funds of the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) based on a decision of the Parliament of

the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program. Further funding was granted by the University of Stirling, Stirling, UK. Thanks also to the staff of the Thuenen Institute of Fisheries Ecology at Ahrensburg for support in fish and laboratory matters.

3.4.5 References

Akinleye, A. O., V. Kumar, H. P. S. Makkar, M. A. Angulo-Escalante, and K. Becker. 2012. *Jatropha platyphylla* kernel meal as feed ingredient for Nile tilapia (*Oreochromis niloticus* L.): growth, nutrient utilization and blood parameters. Journal of Animal Physiology and Animal Nutrition 96:1–11.

Cho, C. Y. and S. J. Slinger. 1979. Apparent digestibility measurement in feedstuffs for rainbow trout. pp. 239–247 in: Finfish Nutrition and Fish Feed Technology Volume II. (J.E. Halver and K Tiews, eds.). Hamburg: Bundesforschungsanstalt fuer Fischerei/Berlin: Heenemann Verlagsgesellschaft 622 p..

El-Saidy, D. M. S. D., and M. M. A. Gaber. 2003. Replacement of fish meal with a mixture of different plant protein sources in juvenile Nile tilapia, *Oreochromis niloticus* (L.) diets. Aquaculture Research 34:1119–1127.

Fontainhas-Fernandes, A., E. Gomes, M. A. Reis-Henriques, and J. Coimbra. 1999. Replacement of fish meal by plant proteins in the diet of Nile tilapia: digestibility and growth performance. Aquaculture International 7:57–67.

Francis, G., H. P. S. Makkar, and K. Becker. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199:197–227.

Gaye-Siessegger, J., U. Focken, H. Abel, and K. Becker. 2007. Influence of dietary non-essential amino acid profile on growth performance and amino acid metabolism of Nile tilapia, *Oreochromis niloticus* (L.). Comparative Biochemistry and Physiology Part A 146:71–77.

Gaye-Siessegger, J., J. S. O. McCullagh, and U. Focken. 2011. The effect of dietary amino acid abundance and isotopic composition on the growth rate, metabolism and tissue ^{13}C of rainbow trout. British Journal of Nutrition 105:1764–1771.

Hardy, R. W. 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquaculture Research 41:770–776.

Harter, T., F. Buhrke, V. Kumar, U. Focken, H. P. S. Makkar, and K. Becker. 2011. Substitution of fish meal by *Jatropha curcas* kernel meal: Effects on growth performance and body composition of white leg shrimp (*Litopenaeus vannamei*). Aquaculture Nutrition 17:542–548.

Kaushik, S. J., D. Covès, G. Dutto, and D. Blanc. 2004. Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. Aquaculture 230:391–404.

Krome, C., K. Jauncey, and U. Focken. In press. Testing two different phytases in a *Jatrophas curcas* kernel meal based diet for Nile tilapia, *Oreochromis niloticus.* Israeli Journal of Aquaculture.

Kumar, V., H. P. S., Makkar, and K. Becker. 2010. Dietary inclusion of detoxified *Jatropha curcas* kernel meal: effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L. Fish Physiology and Biochemistry, 36:1159–1170.

Kumar, V., H. P. S. Makkar, and K. Becker. 2011a. Detoxified *Jatropha curcas* kernel meal as a dietary protein source: growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings. Aquaculture Nutrition 17:313–326.

Kumar, V., H. P. S. Makkar, and K. Becker. 2011b. Nutritional, physiological and haematological responses in rainbow trout (*Oncorhynchus mykiss*) juveniles fed detoxified *Jatropha curcas* kernel meal. Aquaculture Nutrition 17:451–467.

Kumar, V., Makkar, H. P. S., Devappa, R. K., and K. Becker. 2011c. Isolation of phytate from *Jatropha curcas* kernel meal and effects of isolated phytate on growth, digestive physiology and metabolic changes in Nile tilapia (*Oreochromis niloticus* L.). Food and Chemical Toxicology 49:2144–2156.

Kumar, V., A. O. Akinleye, H. P. S. Makkar, M. A. Angulo-Escalante, and K. Becker. 2012. Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing *Jatropha platyphylla* kernel meal as a protein source. Journal of Animal Physiology and Animal Nutrition 96:37–46.

Li, J. S., J. L. Li, and T. T. Wu. 2009. Effects of non-starch polysaccharides enzyme, phytase and citric acid on activities of endogenous digestive enzymes of tilapia (*Oreochromis niloticus* × *Oreochromis aureus*). Aquaculture Nutrition 15:415–420.

Maga, J. A. 1982. Phytate: Its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis. Journal of Agricultural and Food Chemistry 30:1–9.

Maier, K.J. and R. E. Tullis. 1984. The effects of diet and digestive cycle on the gastrointestinal tract pH values in the goldfish, *Carassius auratus* L., Mozambique tilapia, *Oreochromis mossambicus* (Peters), and channel catfish, *Ictalurus punctatus* (Rafinesque). Journal of Fish Biology 25:151–165.

Makkar, H. P. S. and K. Becker. 2009. *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. European Journal of Lipid Science and Technology 111:773–787.

Mazid, M. A., Y. Tanaka, T. Katayama, M. A. Rahman, K. L. Simpson, and C. O. Chichester. 1979. Growth response of *Tilapia Zillii* fingerlings fed iso-caloric diets with variable protein levels. Aquaculture 18:115–122.

NRC, Committee on the Nutrient Requirements of Fish of the National Research Council, Washington. 1993. Nutrient requirements of fish and shrimp. The National Academic Press.

NRC, Committee on the Nutrient Requirements of Fish and Shrimp of the National Research Council, Washington. 2011. Nutrient requirements of fish and shrimp. The National Academic Press.

Nwanna, L. C., M. Kolahsa, R. Eisenreich, and F. J. Schwarz. 2008. Pre-treatment of dietary plant feedstuffs with phytase and its effect on growth and mineral concentration in common carp (*Cyprinus carpio* L.). Journal of Animal Physiology and Animal Nutrition 92:677–682.

Openshaw, K. 2000. A review of *Jatropha curcas*: an oil plant of unfulfilled promise. Biomass & Bioenergy 19:1–15.

Riche, M., N. L. Trottier, P. K. Ku, and D. L. Garling. 2001. Apparent digestibility of crude protein and apparent availability of individual amino acids in tilapia (*Oreochromis niloticus*) fed phytase pretreated soybean meal diets. Fish Physiology and Biochemistry 25:181–194.

Sajjadi, M., and C. G. Carter. 2004. Effect of phytic acid and phytase on feed intake, growth, digestibility and trypsin activity in Atlantic salmon (*Salmo salar*, L.). Aquaculture Nutrition 10:135–142.

Spinelli, J., C. R. Houle, and J. C. Wekell. 1983. The effect of phytates on the growth of rainbow trout (*Salmo gairdneri*) fed purified diets containing varying quantities of calcium and magnesium. Aquaculture 30:71–83.

Tacon, A. G. J., and M. Metian. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture 285:146–158.

Trosvik, K. A., S. D. Rawles, K. R. Thompson, L. A. Metts, A. Gannam, R. Twibell, and C. D. Webster. 2012. Growth and body composition of Nile tilapia, *Oreochromis niloticus*, fry fed organic diets containing yeast extract and soybean meal as replacements for fish meal, with and without supplemental lysine and methionine. Journal of the World Aquaculture Society 43:635–647.

4 The Role of Phytate as an Anti-nutritional Factor in JKM-based Diets for Tilapia

4.1 Testing two different phytases in *Jatrophas curcas* kernel meal based diets for Nile tilapia, *Oreochromis niloticus*

March – May 2012

Accepted, The Israeli Journal of Aquaculture

Carsten Krome[1,2], Kim Jauncey[1], Ulfert Focken[2]

[1)] Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, UK

[2)] Institute of Fisheries Ecology, Thuenen-Institute, Wulfsdorfer Weg 204, 22926, Ahrensburg, Germany

Keywords: *Jatropha curcas*, *Oreochromis niloticus*, Phytate, Phytase, Growth

Abstract

The current experiment tested JKM as a feedstuff in terms of growth and body composition of *Oreochromis niloticus* and further elucidated the efficacy of two phytases (3-phytase and 6-phytase), supplemented singly or in combination. Five diets were produced, a fishmeal based positive control diet (Diet FM) and 4 diets containing 100% of the protein from JKM. Of the four JKM-based diets, diet Natu contained 2000 U/kg of a 3-phytase, diet Rono 2000 U/kg of a 6-phytase, diet Combined 1000 U/kg of each phytase and diet Control no phytase. Diet FM resulted in better body mass gain than all JKM-based diets, but the difference to diet "Combined" was not significant. JKM-based diets containing one phytase (diet Rono and diet Natu), showed no growth improvement over the Control without phytase. Diet Combined resulted in significantly higher specific growth rates than diet Control ($p = 0.045$). Diet Combined also exhibited improved protein efficiency ratios suggesting better protein utilization in this diet, though these differences were not significant. All

phytase-containing diets showed higher body dry matter and body ash values presumably due to increased bone mineralization. In conclusion, JKM as the major protein source does not result in performance as good as fishmeal. A single type of phytase had no impact, but applying two different types in combination showed an improvement in growth parameters. However, further research is required to confirm these results.

4.1.1 Introduction

In this experiment, a 3-phytase and a 6-phytase were tested separately and in combination in a solely JKM-based, phosphorus-supplemented diet. A diet without phytase served as a negative control and a fish meal-based control diet served as a positive control. The experiment was aimed at finding out whether there is an impact of phytase on protein assimilation and efficiency when feeding JKM-based diets and whether there is a difference in efficacy between the two types of phytase.

4.1.2 Material and methods

Experimental diets

Table 4.1.1 shows the composition of the experimental diets. Five diets were produced: Four JKM-based diets, one without phytase (Control); one with the 3-phytase Natuphos produced by BASF (Natu); one with the 6-phytase Ronozyme produced by DSM (Rono) and one where half the concentration of both phytase types were applied (Combined). The fifth diet was a fishmeal-based control diet (FM). Crystalline amino acids were added to the JKM-based diets to meet the latest nutritional requirement standards (NRC, 2011). The raw materials of the experimental diets were mixed in a standard kitchen blender. Phytases were applied at 2000 U/kg diet (Biswas et al, 2007). In diet Combined both phytases were applied at 1000 U/kg. The enzyme was added to the water before the water was added to the raw materi-

als. Diets were mixed with a standard kitchen machine, pelleted in a meat mincer and subsequently dried at 45°C for 48 hours and during the trial stored at 4°C.

Phytase incubation of diets

To get an idea of the efficiency of phytase in the fish stomach, a stomach environment was simulated *in vitro*. 0.5 g of the respective feed was added to 5 ml of HCl acidified water (pH 3.5) and incubated for 8 hours at 23.5°C on a magnetic stirrer. Phytate was analyzed after the incubation. The conditions were designed to resemble the situation inside the fish´s stomach after the feed is consumed and when most phytate degradation takes place (Maier et al, 1984).

Feeding trial

For each of the five treatments, n = 8 all-male, juvenile tilapia obtained from Til-Aqua, Someren, Netherlands, (initial mean weight = 8.88 g ± 0.55) were reared individually in 40 L tanks. The duration of the feeding trial was 8 weeks. The water temperature of the recirculation system was 23.5°C and water flow was set to 1.5 L/min. Water quality parameters were measured weakly and were below the critical limits.

As a parameter for feed quantity, the metabolic maintenance ration of the fish was calculated (see 2.1.3).

Pre- and post experimental procedures, chemical analyses as well as performance calculations and and equations were conducted as outlined in Chapter 2.

Statistics

Statistica Release 7 was used to perform one-way ANOVA ($p \leq 0.05$) and Tukey's test was applied to test for significant differences between the diets. Percentages were arcsine transformed before analysis. Results from the analysis of variance are presented as ± standard deviation unless otherwise stated.

Table 4.1.1 Composition and proximate analysis of diets fed to Nile tilapia for 8 weeks.

Ingredient (g/100g)	FM	Natu	Rono	Control	Combined
Fish Meal	51.5	0.0	0.0	0.0	0.0
Wheat Meal	34.5	21.7	21.7	21.7	21.7
JKM	0.0	60.4	60.4	60.4	60.4
Sun Flower Oil	4.4	4.5	4.5	4.5	4.5
Fish Oil	0.0	5.2	5.2	5.2	5.2
Cellulose	4.0	0.4	0.4	0.4	0.4
Vitamin Premix[1)]	2.0	2.0	2.0	2.0	2.0
Mineral Premix[2)]	2.0	2.0	2.0	2.0	2.0
Histidine	0.6	0.1	0.1	0.1	0.1
Methionine	0.0	0.8	0.8	0.8	0.8
Lysine	0.0	2.1	2.1	2.1	2.1
Threonine	0.3	0.2	0.2	0.2	0.2
Valine	0.5	0.6	0.6	0.6	0.6
Phenylalanine	0.2	0.0	0.0	0.0	0.0
Phytase Ronozyme (U/kg)	0	0	2000	0	1000
Phytase Natuphos (U/kg)	0	2000	0	0	1000
Analyzed parameters					
Dry Matter	92.9	89.1	88.4	91.9	91.3
Crude Protein	44.1	42.5	43.7	42.4	42.0
Crude Lipid	10.3	10.3	11.6	10.2	10.9
Ash	9.7	9.7	9.7	10.2	9.9
Total P[3)]	1.70	1.37	1.37	1.37	1.37
Phytate-P[4)]	0.06	0.00	0.00	0.51	0.00
Available P[5)]	1.64	1.37	1.37	0.86	1.37

[1)] Vitamin premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg premix, unless otherwise stated): Vitamin A: 500 000 I.E./kg; Vitamin D3: 50 000 I.E./kg; Vitamin E: 2500; Vitamin K3 (as Menadione): 1000; Vitamin B1: 5000; Vitamin B2: 5000; Vitamin B6: 5000; Vitamin B12: 5; Nicotinic acid: 25000; Pantothenic acid: 10000; Folic acid: 1000; Biotin: 250; Choline chloride: 100000; Inositol: 25000; Vitamin C: 20125.

[2)] Mineral premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg premix): Calcium: 122 160; Phosphorus: 83 670; Magnesium: 14 960; Sodium: 18 180; Potassium: 210 250; Sulphur: 15,460; Chlorine: 29 720; Iron: 1220; Manganese: 1000; Zinc: 1630; Copper: 155; Iodine: 120.

[3)] P-content of the diet calculated from P-content of individual feed ingredients determined by ICP-MS

[4)] Phytate content analyzed by HPIC after *in vitro* incubation. Calculation of phytate-P: Phytate-P (%) = analyzed phytate in diet (%) * 0.282

[5)] Available P = Total P – Phytate-P

4.1.3 Results

Of all experimental fish, seven stopped or interrupted feeding during the course of the experiment. Out of these seven, two were obvious phenotypically female. All seven fish were excluded from the analysis. There was no connection between treatment and feed consumption of experimental fish. There was no mortality of experimental fish during the experiment. Figure 4.1.1 and Table 4.1.3 show the increment in body mass of experimental fish. Body mass gain was higher in the fish meal based diet than in all JKM-based diets. Of the JKM-based diets, diet Combined showed highest body mass gain and was the only diet that was not statistically different to the fish meal-based diet ($p \leq 0.05$). FCR was significantly lower in diet FM than in other diets except for diet Combined ($p \leq 0.05$). Feed utilization parameters (Table 4.1.3) show that PER for fish fed diet FM was equal to diet Combined and higher than all other diets, though this difference was not significant. Due to large standard deviations, there were no significant differences observed in PPV and LPV, respectively, between the treatments. Body composition of fish is shown in Table 4.1.2. Dry matter of diet FM and diet Control was significantly lower than in the other treatments. Ash contents, though not significant, were also lower in these diets, while protein content showed the opposite with phytase-treated diets showing a lower content than diet FM and diet Control.

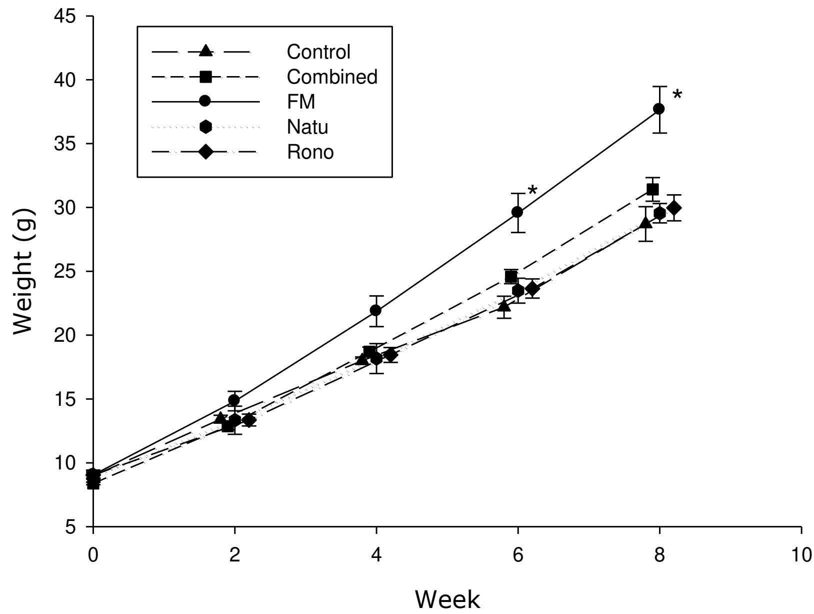


Figure 4.1.1 Growth of *Oreochromis niloticus* fed different experimental diets.

Variances are depicted as ± standard error of the mean. *Significantly different from other treatments ($p \leq 0.05$).

Table 4.1.2 Body composition of Nile tilapia fed different experimental diets for 8 weeks.

Treatment	Dry Matter				Crude Protein				Crude Lipid			Ash			
Initial Fish	25.4	±	2.32		60.1	±	3.71		9.6	±	5.71	21.2	±	3.78	
FM	23.1	±	1.18	a	64.8	±	2.27	a	17.6	±	3.20	15.4	±	0.63	a
Natu	26.0	±	0.78	b	62.0	±	1.98	a,b	16.7	±	3.27	18.5	±	1.39	b
Rono	26.2	±	0.66	b	59.7	±	0.8	b	20.1	±	2.81	17.5	±	0.70	b
Control	24.4	±	1.21	a	64.5	±	1.48	a	15.5	±	2.75	16.9	±	0.89	a,b
Combined	26.0	±	1.08	b	60.0	±	1.72	b	20.1	±	3.34	17.6	±	1.23	b

Values in % DM. Mean values with different superscripts differ significantly from one another ($p \leq 0.05$). No significant differences found for crude lipid content between treatments.

Table 4.1.3 Feed utilization parameters of Nile tilapia fed experimental diets for 8 weeks.

Treatment	IW (g)	FW (g)	BMG	SGR	FCR	MGR	PER	PPV	LPV
FM	9.1 ± 0.69	37.7 ± 4.47 ^{a}	28.6 ± 4.14 ^{a}	2.54 ± 0.21 ^{a}	0.90 ± 0.10 ^{a}	10.64 ± 0.79 ^{a}	2.54 ± 0.29 ^{a}	37.4 ± 5.02	49.1 ± 10.16
Natu	8.9 ± 0.45	29.6 ± 0.75 ^{b}	20.6 ± 0.99 ^{b}	2.13 ± 0.11 ^{b,c}	1.10 ± 0.07 ^{b}	8.89 ± 0.41 ^{b}	2.14 ± 0.14 ^{a,b}	34.3 ± 2.32	44.8 ± 9.85
Rono	9.1 ± 0.51	30.0 ± 2.49 ^{b}	20.9 ± 2.45 ^{b}	2.14 ± 0.16 ^{b,c}	1.10 ± 0.10 ^{b}	8.93 ± 0.66 ^{b}	2.10 ± 0.19 ^{b}	33.4 ± 1.96	52.1 ± 9.66
Control	9.1 ± 0.47	28.7 ± 3.31 ^{b}	19.7 ± 3.16 ^{b}	2.07 ± 0.19 ^{c}	1.15 ± 0.16 ^{b}	8.62 ± 0.83 ^{b}	2.09 ± 0.27 ^{b}	33.6 ± 4.05	39.0 ± 11.83
Combined	8.5 ± 0.49	31.4 ± 2.26 ^{b}	23.0 ± 2.37 ^{a,b}	2.36 ± 0.16 ^{a,b}	1.00 ± 0.10 ^{a,b}	9.71 ± 0.63 ^{a,b}	2.40 ± 0.23 ^{a,b}	37.9 ± 3.85	58.1 ± 12.22

Mean values with different superscript differ significantly from one another ($p \leq 0.05$). No significant differences found between treatments for PPV and LPV. IW, initial weight (g); FW, final weight (g); BMG, body mass gain (g); SGR (%/day), specific growth rate; FCR, feed conversation ratio; MGR (g x $kg^{-0.8}$ day^{-1}), metabolic growth rate; PER, protein efficiency ratio; PPV (%), protein productive value; LPV (%), lipid productive value.

4.1.4 Discussion

Aquaculture diets based on JKM as a protein source have been tested in tilapia (Akinleye et al., 2011; Kumar et al., 2012), carp (Kumar et al., 2010; Kumar et al., 2011a) and rainbow trout (Kumar et al., 2011c). For carp and tilapia or rainbow trout, up to 50% and 62.5% respectively of fishmeal could be replaced by JKM with no reduction in growth. Diets with 75% replacement level in carp showed a reduction in growth compared to the fishmeal control. In these studies, the decrease was attributed to anti-nutritional factors in JKM, predominantly phytate (Kumar et al., 2011a). The present study shows that tilapia fed a 100% JKM-based diet grow significantly slower than when fed fishmeal-based diets despite compensation of essential amino acids (Figure 4.1.1).

As stated, it is undisputed that phytate has adverse effects on absorption of minerals, such as phosphorus, calcium, magnesium and zinc (Storebakken et al., 1998). Treating phytate-containing feedstuffs with commercially available phytase has improved growth and/or protein and mineral availability in several species, such as carp (Baruah et al., 2005; Nwanna et al., 2007; Nwanna et al., 2008) and rainbow trout (Vielma et al., 2004; Wang et al., 2008; Vandenberg et al., 2011). In these experiments, diets were phosphorus-limited and consequently growth was impaired by the assimilation of phosphorus and consequently bone mineralization (Roy et al., 2004). The release of phosphate from phytate through phytase increased phosphate supply and as a secondary effect improved growth and therefore protein utilization. However, this does not solve the question whether phytate itself impairs the assimilation of protein in the digestive tract. Whether there is a direct impact of phytate on nitrogen retention and presumably growth in JKM-based diets was a target of this study. The available phosphorus content in all diets was sufficient for the requirement of tilapia (NRC, 2011).

It has been shown *in vitro* for casein and soybean meal protein in a simulated gastric environment that the protein solubility decreases significantly when phytate is present. Insoluble protein is impossible to absorb, which is why these results suggest a direct influence of phytate on protein digestibility (Kies et al., 2006; Morales et al., 2011). Some *in vivo* studies seem to support these results as shown for rainbow trout (Spinelli et al., 1983), Atlantic salmon (Sajjadi & Carter, 2004), carp (Sardar et al., 2007) or red sea bream (Laining et al., 2011). In these studies, protein digestibility is significantly impaired by the presence of phytate in the diets and this effect is counterbalanced in the diets containing phytase. A different approach was undertaken by Kumar et al. (2011b) who isolated a phytate-rich fraction from JKM and included this in a casein based diet. They found that the phytate-rich fraction significantly decreased PER when added to the diet at 1.5% or 3% respectively. Further, Sajjadi & Carter (2004) observed improved growth when adding 2000 U / kg of phytase to a non-phytate containing diet with sufficient available phosphorus in Atlantic salmon. This suggests a phytate-independent function of phytase. In contrast to these results are various *in vivo* studies demonstrating no improvement effect of phytase on protein digestibility. In a study by Augspurger & Baker (2004), PER of chicks was not affected by the addition of phytase to an amino acid deficient diet. Likewise, parrot fish showed no improvement in PER when phytase was added to a cottonseed and soybean meal containing diet (Lim & Lee, 2009). Cao et al. (2008) demonstrated that phytase leads to higher PER of tilapia at lower available phosphate levels, but has no additional effect when phosphate is supplemented to the required level. No influence on nitrogen retention through phytase addition was reported for soybean meal based diets in tilapia (Riche et al., 2004). Phytase addition may even lead to a decrease of lysine and methionine digestibility (Riche et al., 2001). Four different feedstuffs (isolated soy protein, soybean meal, corn gluten meal and wheat middlings) were tested with phytase on striped bass (*Morone*

saxatilis) without improvement in protein digestibility (Parathryphon & Soares, 2001). The present study did not show significant improvement of a single phytase on growth of tilapia fed JKM-based diets. Neither of the two phytases showed a significant improvement of PER or PPV. However, though not significant, there seems to be a slight improvement in growth when both types of phytase were combined. This increment in growth derives from improved protein utilization as can be seen in a higher PER and PPV of this diet. Reasons for this may lie in the nature of the applied types of phytase. The two phytases applied have slightly different pH optima (Cao et al., 2007). When functioning in an environment like the tilapia stomach with fluctuating pH, using two types of phytase simultaneously could lead to a longer maximum period of action inside the stomach and therefore more effective hydrolysis of phytate-protein complexes respectively (Morales et al., 2013). The significant difference in body dry matter of phytase-containing diets in this trial is most likely due to higher mineral content of the carcass. This is also supported by slightly higher ash values in fish fed these diets. Fox & Davies (2011) have shown that phytase can enhance scale and vertebrae mineralization in rainbow trout fed phytase-containing soybean meal-based diets and Baruah et al. (2007) found that ash content increased significantly when phytase was applied along with citric acid in Rohu carp (*Labeo rohita*).

In conclusion, it can be assumed that despite significantly reducing phytate content, 100% JKM-based diets are not as growth-efficient as fish meal based diets. Further, it can be suggested that applying a single phytase to a diet with sufficient available phosphate has no impact on protein efficiency ratio or protein productive value. Body dry matter and ash contents are significantly increased presumably due to higher bone mineralization in these treatments. There is evidence that applying two different types of phytase simultaneously may be beneficial in terms of growth parameters, but more research in this field is required to confirm these results.

Acknowledgements

The project was partly supported by funds of the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program. Further funding was granted by the University of Stirling, Stirling, UK. A travel grant to Carsten Krome by the Deutsche Akademische Austauschdienst (DAAD) to the ISTA10 conference in Jerusalem, 2013, is also gratefully acknowledged. The help with analysis by Prof. Rodehutscord and staff of the University of Hohenheim, Germany, and William Struthers of the Institute of Aquaculture in Stirling, UK, is well-appreciated. Thanks also to Sybille Fedderke, Anne Beiermeister, Andreas Drahotta and Andreas Willert for helping with the experimental proceedings.

4.1.5 References

Akinleye, A. O., Kumar, V., Makkar, H. P. S., Angulo-Escalante, M. A. and K. Becker, 2011. *Jatropha platyphylla* kernel meal as feed ingredient for Nile tilapia (*Oreochromis niloticus* L.): growth, nutrient utilization and blood parameters. *J. Anim. Physiol. An. N.*, 96:1-11.

Augspurger, N. R. and D. H. Baker, 2004. High dietary phytase levels maximize phytate-phosphorus utilization but do not affect protein utilization in chicks fed phosphorus- or amino acid-deficient diets. *J. Anim. Sci.*, 82:1100–1107.

Baruah, K., Pal, A. K., Sahu, N. P., Jain, K. K., Mukherjee, S. C. and D. Debnath, 2005. Dietary protein level, microbial phytase, citric acid and their interactions on bone mineralization of *Labeo rohita* (Hamilton) juveniles. *Aquac. Res.*, *36*:803–812.

Baruah, K., Sahu, N. P., Pal, A. K., Jain, K. K., Debnath, D. and S. Mukherjee, 2007. Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. *Aquac. Res.*, *38*:109–120.

Biswas, A., Kaku, H., Ji, S., Seoka, M. and K. Takii, 2007. Use of soybean meal and phytase for partial replacement of fish meal in the diet of red sea bream, *Pagrus major*. *Aquaculture*, *267*:284-291.

Cao, L., Wang, W., Yang, C., Yang, Y., Diana, J., Yakupitiyage, A. and Z. Luo, 2007. Application of microbial phytase in fish feed. *Enzyme Microb. Tech.*, *40*:497-507.

Cao, L., Yang, Y., Wang, W. M., Yakupitiyage, A., Yuan, D. R. and J. S. Diana, 2008. Effects of pretreatment with microbial phytase on phosphorous utilization and growth performance of Nile tilapia (*Oreochromis niloticus*). *Aquacult. Nutr.*, *14*:99–109.

Fox, S. W. and S.J. Davies 2011. Corn-expressed dietary phytase augments vertebral and scale mineral content in juvenile rainbow trout (Oncorhynchus mykiss). *Aquacult. Nutr.*, *17*:e840–e852.

Kies, A. K., De Jonge, L. H., Kemme, P. A. and A. W. Jongbloed, 2006. Interaction between protein, phytate, and microbial phytase. *In vitro* studies. *J. Agr. Food Chem.*, *54*:1753–1758.

Kumar, V., Makkar, H. P. S. and K. Becker, 2010. Dietary inclusion of detoxified *Jatropha curcas* kernel meal: effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L. *Fish Physiol. Biochem.*, *36*:1159-70.

Kumar, V., Makkar, H. P. S. and K. Becker, 2011a. Detoxified *Jatropha curcas* kernel meal as a dietary protein source: growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio L.*) fingerlings. *Aquacult. Nutr.*, *17*:313-326.

Kumar, V., Makkar, H. P. S., Devappa, R. K. and K. Becker, 2011b. Isolation of phytate from *Jatropha curcas* kernel meal and effects of isolated phytate on growth, digestive physiology and metabolic changes in Nile tilapia (*Oreochromis niloticus L.*). *Food Chem. Toxicol.*, *49*:2144-2156.

Kumar, V., Makkar, H. P. S. K. Becker, 2011c. Nutritional, physiological and haematological responses in rainbow trout (*Oncorhynchus mykiss*) juveniles fed detoxified *Jatropha curcas* kernel meal. *Aquacult. Nutr.*, *17*:451–467.

Kumar, V., Akinleye, A. O., Makkar, H. P. S., Angulo-Escalante, M. A. and K. Becker, 2012. Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis*

niloticus L.) fed on a diet containing *Jatropha platyphylla* kernel meal as a protein source. *J. Anim. Physiol. An. N.*, 96:1-10.

Laining, A., Ishikawa, M., Koshio, S., Lideman and S. Yokoyama, 2011. Dietary inorganic phosphorus or microbial phytase supplementation improves growth, nutrient utilization and phosphorus mineralization of juvenile red sea bream, *Pagrus major*, fed soybean-based diets. *Aquacult. Nutr., 18*:502-511.

Lim, S.-J. and K.-J. Lee, 2009. Partial replacement of fish meal by cottonseed meal and soybean meal with iron and phytase supplementation for parrot fish *Oplegnathus fasciatus*. *Aquaculture*, *290*:283–289.

Maier, K. J. and R.E. Tullis, 1984. The effects of diet and digestive cycle on the gastrointestinal tract pH values in the goldfish, *Carassius auratus* L., Mozambique tilapia, *Oreochromis mossambicus* (Peters) and channel catfish, *Ictalurus punctatus* (Rafinesque). *J. Fish. Bio.*, *25*:151-165.

Morales, G. A., Moyano, F. J. and L. Marquez, 2011. *In vitro* assessment of the effects of phytate and phytase on nitrogen and phosphorus bioaccessibility within fish digestive tract. *Anim. Feed. Sci. Tech.*, *170*:209–221.

Morales, G. A., Márquez, L., Saenz de Rodrigañez, M., Bermúdez, L., Robles, R. and F.J. Moyano, 2013. Effect of phytase supplementation of a plant-based diet on phosphorus and nitrogen bioavailability in sea bream, *Sparus aurata*. *Aquaculture Nutrition*, Online Version. doi:10.1111/anu.12063

NRC, 2011. *Nutrient Requirements of Fish and Shrimp*. Committee on the Nutrient Requirements of Fish and Shrimp of the National Research Council. The National Academic Press, Washington D.C.

Nwanna, L., Eisenreich, R. and F. Schwarz, 2007. Effect of wet-incubation of dietary plant feedstuffs with phytases on growth and mineral digestibility by common carp (*Cyprinus carpio* L). *Aquaculture*, *271*:461-468.

Nwanna, L. C., Kolahsa, M., Eisenreich, R. and F. J. Schwarz, 2008. Pre-treatment of dietary plant feedstuffs with phytase and its effect on growth and mineral concentration in common carp (*Cyprinus carpio* L.). *J. Anim. Physiol. An. N.*, *92*:677-82.

Papatryphon, E. and J. H. Soares Jr, 2001. The effect of phytase on apparent digestibility of four practical plant feedstuffs fed to striped bass, *Morone saxatilis*. *Aquacult. Nutr.*, *7*:161–167.

Pradhan, S., Naik, S. N., Khan, M. A. I. and P. Sahoo, 2011. Experimental assessment of toxic phytochemicals in *Jatropha curcas*: oil, cake, bio-diesel and glycerol. *J. Sci. Food. Agr.*, *92*:511-519.

Riche, M., Trottier, N. L., Ku, P. K. and L. D. Garling, 2002. Apparent digestibility of crude protein and apparent availability of individual amino acids in tilapia (*Oreochromis niloticus*) fed phytase pretreated soybean meal diets. *Fish Physiol. Biochem.*, *25*:181-194.

Roy, P. K., Witten, P. E., Hall, B. K. and S. P. Lall, 2004. Effects of dietary phosphorus on bone growth and mineralisation of vertebrae in haddock (*Melanogrammus aeglefinus L.*). *Fish Physiol. Biochem.*, *27*:35–48.

Sajjadi, M. and C. G. Carter, 2004. Effect of phytic acid and phytase on feed intake, growth, digestibility and trypsin activity in Atlantic salmon (*Salmo salar, L.). Aquacult. Nutr.*, *10*:135-142.

Sardar, P., Randhawa, H. S., Abid, M. and S. K. Prabhakar, 2007. Effect of dietary microbial phytase supplementation on growth performance, nutrient utilization, body compositions and haemato-biochemical profiles of *Cyprinus carpio* (L.) fingerlings fed soyprotein-based diet. *Aquacult. Nutr.*, *13*:444-456.

Spinelli, J., Houle, C and J. Wekell, 1983. The effect of phytates on the growth of rainbow trout (*Salmo gairdneri*) fed purified diets containing varying quantities of calcium and magnesium. *Aquaculture*, *30*:71-83.

Vandenberg, G. W., Scott, S. L., Sarker, P. K., Dallaire, V. and J. de la Noüe, 2011. Encapsulation of microbial phytase: Effects on phosphorus bioavailability in rainbow trout (*Oncorhynchus mykiss*). *Anim. Feed Sci. Tech.*, *169*:230-243.

Wang, F., Yang, Y.-H., Han, Z.-Z., Dong, H.-W., Yang, C.-H. and Z.-Y. Zou, 2008. Effects of phytase pretreatment of soybean meal and phytase-sprayed in diets on growth appar-

ent digestibility coefficient and nutrient excretion of rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquacult. Int.*, *17*:143-157.

4.2 Influence of phytase on growth and mineral retention of tilapia fed non-phosphate supplemented *Jatropha curcas* kernel meal-based diets

October 2012 – January 2013

Carsten Krome[1,2], Kim Jauncey[2], Simon Paysen[1], Sibylle Fedderke[1], Ulfert Focken[1]

[1)] Thuenen Institute of Fisheries Ecology, Wulfsdorfer Weg 204, 22926, Ahrensburg, Germany,

[2)] Institute of Aquaculture, University of Stirling, Stirling, UK

Abstract

Detoxified *Jatropha curcas* kernel meal is a high-potential feedstuff due to its high protein content and balanced amino acid composition. However, substantial amounts of phytate in the meal may adversely influence nutrient and mineral availability. To identify the impact of phytate and phytase on detoxified *Jatropha curcas* kernel meal in tilapia (*Oreochromis niloticus*), supplemented diets were produced containing 100% JKM as a protein source: Diet Control (no additions), diet Jatphos (1.5% NaH_2PO_4), diet Jatphyt (10,000 U / kg phytase) and Jatphosphyt (1.5% NaH_2PO_4 + 10,000 U / kg phytase). Growth parameters, body composition as well as mineral utilization of experimental fish were measured after feeding each diet to four replicates of each treatment for eight weeks. Though not significant, growth performance parameters such as weight gain, specific growth rate and feed conversion ratio were better in all treatment diets compared to the control diet. Fish body composition showed higher total ash, phosphorus and calcium values in treatment diets compared to the control. Phosphorus retention and phosphorus load of effluent water were significantly higher and lower respectively in diet Jatphyt than in all other diets. In conclusion, phytase applied to JKM-based diets shows a tendency to benefit growth of tilapia and significantly improves mineral utilization, specifically phosphorus, which contributes to lower environmental impact of effluent water.

4.2.1 Introduction

In this experiment, phytase is tested in combination with the novel feedstuff JKM to assess impacts on growth, nutrient and mineral retention in tilapia. It further addresses the question whether additional phosphate supplementation is necessary if phytase is applied.

4.2.2 Material and methods

Experimental diets

Detoxified JKM was obtained from JatroSolutions GmbH (Hohenheim, Germany). Four isoenergetic and isonitrogenous (19.7 kJ/g; 35% protein) diets were designed with JKM as the only protein source. Insufficient lysine and methionine levels were supplemented with crystalline amino acids according to current standards of the NRC, 2011. Diet composition is shown in Table 4.2.1. A diet without additional phosphate served as a negative control (Control). Diet “Jatphyt” contained 10,000 U / kg phytase (Natuphos 5000G, BASF, Ludwigshafen, Germany). A positive control diet (Jatphos) contained phosphate supplementation in the form of 1.5% NaH_2PO_4. To test for potential synergies between phosphate and phytase, another diet was produced that contained phytase and additional phosphate (“Jatphosphyt)”. All diets contained 1% TiO_2 as a digestibility marker. Diets were mixed in a kitchen blender. Phytase was blended with water (40% of diet weight) before added to the diet and extruded with a kitchen meat mincer to pellets of 2 mm diameter. Diets were dried in at 45°C for 48h in a ventilated drying chamber and then stored at 4°C.

Experimental design

Four treatments, each with N = 4 replicates and each replicate containing four fish were randomly split up into sixteen 45 L aquaria, connected to a recirculation system. Fish were given a one week acclimatization period during which they were fed a standard, commercial tilapia diet. Water temperature and flow was set to 26°C and

1.5 L / min respectively. Water quality was monitored on a weekly basis. Water oxygen concentration and pH varied between 7-9 mg / L and 6.7-7.7 respectively throughout the duration of the experiment. The system was subjected to a light / dark photoperiod of 12h/12h. Starting weight of fish was 5.7 ± 0.09 g. Fish were weighed every two weeks and feeding ratios were adapted each time.

As a parameter for feed quantity, the metabolic maintenance ration of the fish was calculated (see 2.1.3).

Pre- and post experimental procedures, chemical analyses as well as performance calculations and and equations were conducted as outlined in Chapter 2.

Statistics

One-way ANOVA ($p < 0.05$) was used to analyze differences between all treatments. Tukey HSD test was applied to test for differences between means and considered significant at $p \leq 0.05$. Percentages were arcsine transformed before analysis. Statistics were conducted with Statistica 8 software. Values are expressed as means ± standard deviation.

Table 4.2.1 Composition of diets containing no addition, phytase and / or phosphate addition fed to Nile tilapia for 8 weeks.

Ingredient (g/100g)	Control	JATphyt	JATphos	JATphosphyt
JKM	53.2	53.2	53.6	53.6
Wheat Meal	32.0	32.0	30.0	30.0
Sun Flower Oil	4.30	4.30	4.40	4.40
Fish Oil	5.00	5.00	5.00	5.00
NaH_2PO_4	0.00	0.00	1.50	1.50
Vitamin Premix[1)]	2.00	2.00	2.00	2.00
Mineral Premix[2)]	2.00	2.00	2.00	2.00
Lysine	0.46	0.46	0.46	0.46
Methionine	0.03	0.03	0.03	0.03
Phytase[3)] (U / kg)	0.00	10000	0.00	10000
TiO_2	1.00	1.00	1.00	1.00
Proximate Analysis (g/100g)				
Dry matter	93.4	93.8	94.8	94.9
Crude protein	36.4	36.0	35.4	35.5
Crude fat	10.7	10.5	10.4	10.5
Ash	11.3	12.4	11.1	12.0
Calcium	1.04	1.09	1.01	1.08
Phosphorus	1.21	1.17	1.61	1.57
Zinc	1.30	1.26	1.34	1.30
Magnesium	0.83	0.87	0.80	0.87
Phytate	8.42	3.14	7.35	3.32

[1)] Vitamin premix for fish supplied by Altromin Spezialfutter GmbH. Composition of vitamin mix (mg/kg, unless otherwise stated): Vitamin A: 500,000 I.E./kg; Vitamin D3: 50,000 I.E./kg; Vitamin E:2500; Vitamin K3 (as Menadione): 1000; Vitamin B1: 5000; Vitamin B2:5000; Vitamin B6: 5000; Vitamin B12: 5; Nicotinic acid: 25000; Pantothenic acid: 10000; Folic acid: 1000; Biotin: 250; Choline chloride: 100000; Inositol: 25000; Vitamin C: 20125.
[2)] Mineral premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg mix): Calcium: 122,160; Magnesium: 14,960; Sodium: 18,180; Potassium: 210,250; Sulfur: 15,460; Chlorine: 29,720; Iron: 1220; Manganese: 1000; Zinc: 1630; Copper: 155; Iodine: 120.
[3)] Natuphos 5000L, 3-Phytase (EC 3.1.3.8), BASF Ludwigshafen, Germany.

4.2.3 Results

Phytate content of feed containing phytase was lower than phytate content in diets without phytase Table 4.1.2. Fish growth is shown in Table 4.2.2. Fish fed control diets grew slower than all treatment diets. Due to large standard deviations, these differences were not significant. BMG, SGR and MGR were higher in treatment di-

ets, while FCR was lower. PER was slightly higher in treatments diets than in the control diet, but also not significant (Table 4.2.3).

Table 4.2.2 Body mass gain of Nile tilapia fed JKM based diets with and without phytase and / or phosphate addition.

Treatment	IW			FW			BMG			BMG (%)		
Control	5.9	±	0.01	13.2	±	1.41	7.4	±	1.41	125.5	±	23.9
Jatphyt	5.8	±	0.11	14.8	±	2.03	9.1	±	2.14	157.8	±	40.2
Jatphos	5.7	±	0.07	14.2	±	0.78	8.5	±	0.78	153.4	±	40.4
Jatphosphyt	5.8	±	0.13	15.2	±	1.29	9.3	±	1.26	160.6	±	21.5

IW, initial weight (g); FW, final weight (g); BMG, body mass gain (g); BMG, body mass gain (%)

Table 4.2.3 Feed utilization parameters of Nile tilapia fed JKM based diets with and without phytase and / or phosphate addition for 8 weeks.

Treatment	SGR			FCR			MGR			PER			PPV			LPV		
Control	1.44	±	0.19	3.10	±	0.44	5.26	±	0.68	1.31	±	0.29	24.1	±	2.90	22.9	±	7.29
Jatphyt	1.67	±	0.28	2.76	±	0.36	6.35	±	1.07	1.49	±	0.32	26.0	±	3.64	22.0	±	6.57
Jatphos	1.63	±	0.10	2.69	±	0.33	6.15	±	0.37	1.47	±	0.13	25.0	±	4.69	28.6	±	5.44
Jatphosphyt	1.71	±	0.14	2.65	±	0.23	6.25	±	0.80	1.62	±	0.23	28.0	±	3.15	34.1	±	9.02

BMG, body mass gain (g); SGR, specific growth rate (%/day); FCR, feed conversation ratio; MGR, metabolic growth rate (g x $kg^{0.8}$ day^{-1}); PER, protein efficiency ratio; PPV, protein productive value (%); LPV, lipid productive value (%); EPV, energy productive value (%).
No significant difference detected between treatments (Tukey HSD, $p \leq 0.05$)

Body composition is shown in Table 4.2.4. Body dry matter content is significantly higher in diet Jatphosphyt than in initial fish, but equal to all other treatments diets. Inversely, protein content is lower in this diet than in initial fish. All treatment diets had higher ash content, phosphorus, calcium and magnesium content than the control diet, though none of these values were significant. There was no difference in zinc content between the treatments.

Phosphorus retention was significantly higher in diet Jatphyt than in all other diets (Table 4.2.5). Calcium retention was higher in treatment diets than in the control diet, however, only diet Jatphosphyt was significant.

Phosphorus load was lower of diet Jatphyt than all other diets, however, only differences to diets Jatphos and Jatphosphyt were significant (Table 4.2.6). There were no differences of nitrogen-load between the treatments.

Table 4.2.4 Body composition of of Nile tilapia fed JKM based diets with and without phytase and / or phosphate addition for 8 weeks.

Treatment	Dry Matter %	Crude Protein %	Crude Lipid %	Ash %	Phosphorus %	Calcium %	Magnesium mg/g	Zinc µg/g
Initial Fish	23.9 ± 0.49 ^{a}	55.0 ± 1.27 ^{a}	28.3 ± 0.96	14.7 ± 1.31	2.67 ± 0.23	4.48 ± 0.46	1.33 ± 0.09	77.6 ± 6.1
Control	26.1 ± 1.10 ^{a,b}	53.1 ± 1.51 ^{a,b}	27.2 ± 2.30	13.8 ± 0.70	2.21 ± 0.27	3.60 ± 0.63	1.25 ± 0.18	66.0 ± 11.2
JATphyt	25.9 ± 1.20 ^{a,b}	51.8 ± 1.90 ^{a,b}	25.0 ± 2.48	15.7 ± 1.05	2.52 ± 0.26	4.20 ± 0.44	1.40 ± 0.18	61.6 ± 5.7
JATphos	26.6 ± 0.54 ^{a,b}	52.4 ± 0.97 ^{a,b}	26.2 ± 2.26	15.7 ± 0.56	2.53 ± 0.33	4.21 ± 0.63	1.38 ± 0.23	67.1 ± 4.2
JATphosphyt	27.9 ± 2.15 ^{b}	50.1 ± 2.52 ^{b}	27.9 ± 3.63	15.7 ± 1.31	2.59 ± 0.22	4.40 ± 0.31	1.43 ± 0.15	64.0 ± 4.2

Values are based on dried, homogenized fish samples. Mean values with same superscript differ significantly from one another ($p \leq 0.05$)

Table 4.2.5 Retention of selected minerals of Nile tilapia fed JKM based diets with and without phytase and / or phosphate addition for 8 weeks.

Diet	Phosphorus	Calcium	Magnesium	Zinc
Control	48.9 ± 7.41[a]	88.8 ± 22.8[a]	4.60 ± 0.80	14.3 ± 4.98
Jatphyt	73.8 ± 14.1[b]	140.6 ± 26.8[a]	6.42 ± 1.69	14.2 ± 2.50
Jatphos	58.0 ± 11.4[a]	139.7 ± 32.8[a]	5.97 ± 1.40	17.8 ± 2.63
Jatphosphyt	59.8 ± 12.5[a]	151.4 ± 28.3[b]	6.48 ± 1.46	16.2 ± 3.14

Values in %. Mean values with different superscript differ significantly from one another ($p \le 0.05$)

Table 4.2.6 Nitrogen and phosphorus load of effluent water from tilapia fed JKM based diets with and without phytase and / or phosphate for 8 weeks.

Diet	Phosphorus	Nitrogen
Control	27.1 ± 3.51[a,b]	875.7 ± 139.6
Jatphyt	22.4 ± 2.97[a]	783.7 ± 103.0
Jatphos	31.1 ± 4.74[b]	720.9 ± 118.7
Jatphosphyt	31.3 ± 3.60[b]	736.0 ± 76.5

Values in g / kg. Mean values with different superscript differ significantly from one another ($p \le 0.05$)

4.2.4 Discussion

There are numerous examples for successful application of phytase in non-phosphate supplemented diets improving phosphorus availability, growth or both. Works include phytase application in diets for tilapia (Liebert & Portz, 2005), rainbow trout (Lanari, 1998, Wang et al., 2008); pangasius (Debnath et al., 2005), sea bream (Laining at al., 2011) or salmon (Denstadli et al., 2007). For agastric fish, like carp, it has been shown to be more effective to pre-incubate the feedstuffs with phytase before application as the environment in the digestive tracts is unsuitable for most commercially available phytase as these function best in slightly acidic environments (Nwanna et al., 2007; Nwanna et al., 2008; Cao et al., 2007). Cao et al. (2008) presented results that demonstrate the efficiency of phytase in all-plant-based diets for tilapia when these were preincubated with phytase. Diets contained soybean meal

and corn gluten meal as the main protein sources and phytate levels in these diets were low (0.3%). The trial included several treatments from 100% required inorganic phosphate addition down to 0% phosphate addition. A significant effect in growth compared to non-phytase treated diet was observed if at least 50% of the original amount of inorganic phosphorus was added on top of phytase. Unlike soybean meal and corn gluten meal, JKM has a very high content of phytate and therefore bound phosphate. Thus, we expected phytase to release sufficient available phosphate to the fish, which is why it was decided not to gradually lower the amount of inorganic phosphate, but only use a 100% and 0% inorganic phosphate treatment with and without phytase. The concentration of phytase applied here (10,000 U / kg) is higher than the ones used in a commercial scenario or in previous publications, which are between 250 and 1500 U / kg (Cao et al., 2007). By choosing the high concentration in the present trial, the authors wanted to ensure maximum hydrolysis of phytate. JKM has a very high phytate content compared to other oilseed meals, which lie usually between 0.2 % and 1 % (Eeckhout & de Paepe, 1994), compared to 9.1% in JKM (Kumar et al., 2010). Phytase addition in the present trial reduced the initial phytate content in the diet by around 62.5%, which is similar to what was achieved in a pre-treatment process of a diet based on soy protein concentrate and wheat meal by Denstadli et al. (2007). Further work is required in order to further reduce phytate content in JKM.

Analysis of growth parameters of this trial revealed that there were no significant differences between treatments. This, however, may only be due to large standard deviations within a treatment as a clear improvement of SGR, BMG, FCR, MGR could be seen for all treatments over the control diet. PER also shows a higher val-ue for these treatments, which suggests that protein utilization is affected by phytase in the case of diet Jatphyt (1.49 ± 0.32 compared to 1.31 ± 0.29 in diet control). There are two different scenarios, which may be suggested in this context:

1) Growth is phosphorus-limited and phytase releases additional phosphate from phytate. This additional phosphate improves bone and scale mineralization and as a secondary effect, this leads to better growth and therefore protein utilization.
2) Phytase directly breaks up protein-phytate bonds in the stomach, thereby improving amino acid supply for the fish.
Whether phytate has an influence on amino acid availability, could not be clarified from the given data. The digestibility analysis with TiO_2 was disturbed in feed and faeces probably due to a JKM-specific component interfering with the H_2O_2 reaction of the acid-digested material (Richter et al., 2003). The addition of inorganic phosphate alone (diet Jatphos) seems to improve PER (1.47 ± 0.13), which would tend towards scenario 1), however, a slight improvement compared to diet Jatphos can be observed if additional phytase is added (diet Jatphosphyt, 1.62 ± 0.23). Generally, the question whether phytase has an impact on protein digestibility and consequently growth has not yet been fully answered. While *in vitro* the forming of protein-phytate complexes in an acidic environment has been demonstrated (Morales et al., 2011), it is still under debate whether these effects also take place *in vivo*. Several publications underline the positive effect of phytase on protein availability *in vivo* for other fish (Vandenberg et al., 2001; Vielma et al., 2004; Liu et al., 2011), while for tilapia these results could not be confirmed (Riche et al., 2001). Further research needs to be conducted in this respect.
On an absolute scale growth of fish in the present trial was very slow. Kumar et al. (2012), observed SGR-values of 1.9 ± 0.05 %/day and FCR-values of 1.7 in tilapia fed diets replacing 62.5% of fishmeal with JKM. FCR in their trial was clearly better observed here. Despite having supplemented deficient amino acids of the diet applied here according to the recent standards of the NRC (2011), it may have been the case that these supplementations were not sufficient. Another possibility is that 100% replacement of fish meal may lead to other anti-nutritional factors crossing a

critical concentration (Francis et al., 2001). Further, fish meal may contain factors that positively influence growth that are not present in JKM, such as the aminosulfonic acid taurine (Gaylord et al., 2007). The deficiencies in growth were also reflected by the generally high fat contents found in fish of all the present treatments. While Akinleye et al. (2011) observed average values around 18% crude fat for tilapia feeding on a diet of which also 62.5% of fishmeal was replaced by JKM, our values were between 25% and 28% (DM) dependent on treatment. Overly high fat contents may be a sign of a lack of one or more essential amino acids. If an amino acid is deficient, the energy delivered by the provided diet in form of carbohydrates or proteins cannot be transformed into muscular growth and is instead stored as additional fat tissue.

Body composition was not significantly different between the dietary treatments. Diet Jatphosphyt, the diet with presumably the highest content of available phosphate, showed significantly higher body dry matter and lower body protein than the initial fish, which may be contributed to differences to the diet fed to experimental fish before trial start. Ash content of diets containing either additional phosphorus or phytase was higher than ash content of the control diet. This was reflected in higher body phosphorus, calcium and magnesium. There were no differences in body zinc concentrations. Initial fish were similar in their body ash and mineral contents to all treatments but the control. No significant differences between phytase and non-phytase treated diets for body dry matter, protein or fat in tilapia fed a mixed diet containing largely soybean meal, rice bran and cassava could be observed by Tudkaew et al. (2008). In this work, body ash and phosphorus content was significantly higher in phytase treated diets, which is similar to the present results.

Nutrient retention was significantly influenced by phytase as can be seen in the higher retentions of phosphorus and calcium in diet Jatphyt (73.8 ± 14.1%) over the control diet (48.9 ± 7.41%). Fortes-Silva et al. (2001) observed higher bone phos-

phorus and calcium contents due to phytase treatment of a soybean meal containing diet in sea bass. Comparable to our study, calcium content of bone increased by a higher degree due to phytase treatment than did the phosphate content. Fish maintain a constant Ca:P ratio in their bones varying between 2:1 and 1.5:1 (Roy et al., 2004). Therefore if phosphate uptake and consequently bone mineralization is promoted, calcium uptake will increase within this ratio. The ratio of the increased amount of calcium to phosphorus between diet Control and the treatment diets in the present data reflect the 2:1 ratio observed by Roy et al. Teleost fish possess calcium transport cells in the gills (ionocytes) allowing them to transport calcium against a concentration gradient (Flik et al., 1993). For cod it was shown that only about 30% of calcium is taken up through the intestine while the rest is taking place at the gills (Sundell et al., 1988). Aside intestine and gills, single ionocytes scattered on the fish skin of tilapia have also shown to contribute to calcium uptake (McCormick et al., 1991). This may explain why the present data showed calcium retention above 100% for diets containing either additional phosphorus, phytase or both.

Along with improved phosphorus retention, the phosphorus load to the water was significantly reduced in the present experiment. Especially in culture operations, where the diet has a high natural phosphorus content -as is the case with JKM- not having to add additional phosphorus can have a profound effect on water quality and therefore yields and effluent pollution. Sugiura et al. (2001) found a reduction of 95%-98% of phosphorus discharge in rainbow trout when fed a low-ash, phytase-containing diet compared to a commercial diet. In the present experiment, the treatment containing phytase decreased phosphorus discharge about 28.0%-28.7% compared to diets containing additional phosphorus. Higher reductions of phosphorus load may be possible if the remaining phytate content of the phytase-containing diets can be lowered further.

Conclusion

The experiment has shown that fish fed a JKM-based diet without additional phosphate display an increase in phosphorus and calcium retention if phytase is added to the diet. The increase is equal to the increase achieved if inorganic phosphate is added. Inorganic phosphate in combination with phytase showed no additional effect. Further, the phosphorus load into the water could be significantly reduced through phytase. It could therefore be established that JKM-based diets do not need additional phosphate if phytase is added. This lowers costs and moreover minimizes environmental damage. To our knowledge this is the first work showing that phytase can efficiently be applied in JKM-based diets.

Acknowledgements

The project was partly supported by funds of the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program. Further funding was granted by the University of Stirling, Stirling, UK. Thanks also to the staff of the Thuenen Institute in Ahrensburg for support in fish and laboratory matters.

4.2.5 References

Akinleye, A. O., Kumar, V., Makkar, H. P. S., Angulo-Escalante, M. A., & Becker, K. (2011). *Jatropha platyphylla* kernel meal as feed ingredient for Nile tilapia (*Oreochromis niloticus* L.): growth, nutrient utilization and blood parameters. *Journal of Animal Physiology and Animal Nutrition*, *96*(1), 1–11.

Cao, L., Wang, W., Yang, C., Yang, Y., Diana, J., Yakupitiyage, A., Luo, Z. (2007). Application of microbial phytase in fish feed. *Enzyme and Microbial Technology*, *40*(4), 497–507.

Cao, L., Yang, Y., Wang, W. M., Yakupitiyage, A, Yuan, D. R., & Diana, J. S. (2008). Effects of pretreatment with microbial phytase on phosphorous utilization and growth performance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture Nutrition*, *14*(2), 99–109.

Cho, C., & Slinger, S. (1979). Apparent digestibility measurement in feedstuffs for rainbow trout. *Finfish Nutrition and Fishfeed Technology*, *2*, 239–247.

Debnath, D., Pal, A. K., Sahu, N. P., Jain, K. K., Yengkokpam, S., & Mukherjee, S. C. (2005). Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. *Aquaculture Research*, *36*(2), 180–187.

Denstadli, V., Storebakken, T., Svihus, B., & Skrede, A. (2007). A comparison of online phytase pre-treatment of vegetable feed ingredients and phytase coating in diets for Atlantic salmon (*Salmo salar* L.) reared in cold water. *Aquaculture*, *269*(1-4), 414–426.

Eeckhout, W., & De Paepe, M. (1994). Total phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs. *Animal Feed Science and Technology*, *47*(1-2), 19–29.

Flik, G., & Verbost, P. M. (1993). Calcium transport in fish gills and intestine. *Journal of Experimental Biology*, *184*, 17–29.

Francis, G., Makkar, H. P. S., & Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, *199*(3-4), 197–227.

Fortes-Silva, R., Sánchez-Vázquez, F. J., & Martínez, F. J. (2011). Effects of pretreating a plant based diet with phytase on diet selection and nutrient utilization in European sea bass. *Aquaculture*, *319*(3-4), 417–422.

Gibson Gaylord, T., Barrows, F. T., Teague, A. M., Johansen, K. a., Overturf, K. E., & Shepherd, B. (2007). Supplementation of taurine and methionine to all-plant protein diets for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *269*(1-4), 514–524.

Kumar, V., Makkar, H. P. S., & Becker, K. (2010). Dietary inclusion of detoxified *Jatropha curcas* kernel meal: effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L. *Fish Physiology and Biochemistry*, *36*(4), 1159–1170.

Kumar, V., Akinleye, A. O., Makkar, H. P. S., Angulo-Escalante, M. A., & Becker, K. (2012). Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing *Jatropha platyphylla* kernel meal as a protein source. *Journal of Animal Physiology and Animal Nutrition*, *96*(1), 37–46.

NRC, Committee on the Nutrient Requirements of Fish and Shrimp of the National Research Council. (2011). *Nutrient Requirements of Fish and Shrimp*. The National Academic Press, Washington.

Nwanna, L. C., Kolahsa, M., Eisenreich, R., & Schwarz, F. J. (2008). Pre-treatment of dietary plant feedstuffs with phytase and its effect on growth and mineral concentration in common carp (*Cyprinus carpio* L.). *Journal of Animal Physiology and Animal Nutrition*, *92*(6), 677–682.

Lanari, D. (1998). Use of nonlinear regression to evaluate the effects of phytase enzyme treatment of plant protein diets for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *161*(1-4), 345–356.

Liebert, F., & Portz, L. (2005). Nutrient utilization of Nile tilapia fed plant based low phosphorus diets supplemented with graded levels of different sources of microbial phytase. *Aquaculture*, *248*(1-4), 111–119.

Liu, L. W., Su, J., & Luo, Y. (2011). Effect of partial replacement of dietary monocalcium phosphate with neutral phytase on growth performance and phosphorus digestibility in gibel carp, *Carassius auratus gibelio* (Bloch). *Aquaculture Research*, 1-10.

McCormick, S. D., Hasegawa, S., & Hirano, T. (1992). Calcium uptake in the skin of a freshwater teleost. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(8), 3635–3638.

Morales, G. A., Moyano, F. J., & Marquez, L. (2011). *In vitro* assessment of the effects of phytate and phytase on nitrogen and phosphorus bioaccessibility within fish digestive tract. *Animal Feed Science and Technology*, *170*(3-4), 209–221.

Nwanna, L., Eisenreich, R., & Schwarz, F. (2007). Effect of wet-incubation of dietary plant feedstuffs with phytases on growth and mineral digestibility by common carp (*Cyprinus carpio* L). *Aquaculture*, *271*(1-4), 461–468.

Nwanna, L. C., Kolahsa, M., Eisenreich, R., & Schwarz, F. J. (2008). Pre-treatment of dietary plant feedstuffs with phytase and its effect on growth and mineral concentration in common carp (*Cyprinus carpio* L.). *Journal of Animal Physiology and Animal nutrition*, *92*(6), 677–682.

Riche, M., Trottier, N. L., Ku, P. K., & Garling, D. L. (2001). Apparent digestibility of crude protein and apparent availability of individual amino acids in tilapia (*Oreochromis niloticus*) fed phytase pretreated soybean meal diets. *Fish Physiology and Biochemistry*, *25*(3), 181–194.

Richter, H., Lueckstaedt, C., Focken, U., & Becker, K. (2003). Evacuation of pelleted feed and the suitability of titanium (IV) oxide as a feed marker for gut kinetics in Nile tilapia. *Journal of Fish Biology*, *63*(5), 1080–1099.

Roy, P. K., Witten, P. E., Hall, B. K., & Lall, S. P. (2004). Effects of dietary phosphorus on bone growth and mineralisation of vertebrae in haddock (*Melanogrammus aeglefinus* L.). *Fish Physiology and Biochemistry*, *27*(1-2), 35–48.

Sundell, B. Y. K., & Bjornsson, B. T. (1988). Kinetics of calcium fluxesacross the intestinal mucosa of the marine teleost, *Gadus morhua*, measured using an *in vitro* perfusion method. *Journal of Experimental Biology*, *140*, 171–186.

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.

Storebakken, T., Shearer, K. D., & Roem, A. J. (2000). Growth, uptake and retention of nitrogen and phosphorus, and absorption of other minerals in Atlantic salmon *Salmo salar* fed diets with fish meal and soy-protein concentrate as the main sources of protein. *Aquaculture Nutrition*, *6*(2), 103–108.

Tudkaew, J., Gabaudan, J., & Phromkunthong, W. (2008). The supplementation of phytase Ronozyme P on the growth and the utilisation of phosphorus by sex-reversed red tilapia (*Oreochromis niloticus* Linn.). *Songklanakarin Journal of Science and Technology*, *30*(1), 17–24.

Vandenberg, G. W., Scott, S. L., & de la Noüe, J. (2011). Factors affecting nutrient digestibility in rainbow trout (*Oncorhynchus mykiss*) fed a plant protein-based diet supplement with microbial phytase. *Aquaculture Nutrition*, *18*(4), 369–379.

Vielma, J., Ruohonen, K., & Peisker, M. (2002). Dephytinization of two soy proteins increases phosphorus and protein utilization by rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, *204*(1-2), 145–156.

Vielma, J., Ruohonen, K., Gabaudan, J., & Vogel, K. (2004). Top-spraying soybean meal-based diets with phytase improves protein and mineral digestibilities but not lysine utilization in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*, *35*(10), 955–964.

Wang, F., Yang, Y.-H., Han, Z.-Z., Dong, H.-W., Yang, C.-H., & Zou, Z.-Y. (2008). Effects of phytase pretreatment of soybean meal and phytase-sprayed in diets on growth, apparent digestibility coefficient and nutrient excretion of rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquaculture International*, *17*(2), 143–157.

4.3 Phytate analysis, *in vitro* and *in vivo* phytase application in *Jatropha curcas* kernel meal

August 2011 – May 2013
Carsten Krome[1,2], Kim Jauncey[2], Gertrud Lohaus[3], Ulfert Focken[1]
1) Thuenen-Institute of Fisheries Ecology, Ahrensburg, Germany
2) Institute of Aquaculture, Stirling, U.K.
3) Institute of Molecular Plant Science, Bergische University Wuppertal, Germany

Abstract

Studies on different photometrical methods for phytate analysis as well as phytase application *in vitro* and *in vivo* for *Jatropha curcas* kernel meal (JKM) have been conducted with the following outcome:

1) Several frequently-used methods for phytate analysis involving the Wade-reagent as a photo indicator severely overestimate the true phytate content of JKM and should not be used despite their comparatively convenient applicability.

2) The reasons for the overestimation of these methods are other anions of the acid extract of JKM, predominantly oxalate. Therefore, the discussed methods should not be used for determination of phytate in any plants that contain significant amounts of oxalate.

3) *In vitro* incubation of JKM with 2000 U / kg phytase at pH 4.5 completely erases phytate, as well as other inositol phosphates (IPs) from the meal. Incubation at the same pH without phytase addition also led to complete phytate eradication from the meal, but left significant amounts of IP5 and IP4 (0.43% and 0.47%, respectively).

4) Feeding Nile tilapia (*Oreochromis niloticus*) with a phytase containing JKM-based diet leads to a significant reduction of about 56% of phytate, 31% of IP5 and 100% of IP4 in the faeces compared to a control diet. This goes along with significantly lower P-, Ca- and Zn-content in the faeces and therefore presumably better assimi-

lation of these minerals. However, it also shows that *in vivo* administration of phytase does not hydrolyze IPs as effectively as phytase-incubation.

4.3.1 Introduction

Various authors have measured the concentration of phytate in JKM and results obtained in these measurements were between 7% and 9% (Aderibigbe et al., 1997; Makkar et al., 1997; Martinez-Herrera et al. 2006; Kumar et al., 2010, 2012a; Pradhan et al., 2011; Akinleye et al., 2011; Saetae & Suntornsuk, 2011, Xiao et al., 2011, Harter et al. 2011, Devappa et al., 2012). This is extraordinarily high compared to other oilseed plants or plant feedstuffs (Ravindran et al., 1994). The photometric method applied in these works was developed by Latta and Eskin (1980) and refined by Vaintraub and Lapteva (1988). It involves the decoloration of a Fe^{2+}-sulfosalicylic acid complex (Wade reagent, Wade, 1953) as the phytate replaces the sulfosalicylic acid at the binding site of the Fe^{2+}-ion. Benefits of these methods are time efficiency and simplicity.

This work is structured in four parts:

1. Evaluation of different photometric methods for phytate determination. Three photometric methods all based on the decolorisation of the Wade reagent are compared in order to evaluate their applicability for JKM. As a reference method, high pressure ion chromatography (HPIC) is applied.

2. Identification of factors influencing the photometric determination of phytate. The content of various anions of JKM extract is measured by HPIC. The most predominant anions of the extract are evaluated in terms of interference with the Wade reagent and therefore their influence on accurate phytate analysis.

3. *In vitro* degradation of phytate through phytase in JKM. The degradation of phytate by phytase releases phosphate, which is thereby made available for digestion in farmed animals. It is therefore possible to minimize crystalline phosphate ad-

dition and lower phosphate release through faeces to the environment, significantly decreasing environmental pollution. Commercial phytase products are established in the markets of poultry and swine culture and are applied more and more in aquaculture as well (Kumar et al., 2012b). For JKM, the efficiency of phytase to release phosphate has not yet been demonstrated.

4. Effect of phytase application on the phytate and mineral composition of faeces of Nile tilapia (*Oreochromis niloticus*) fed JKM-based diets. A feeding trial is conducted to evaluate whether the effects of phytase degradation observed *in vitro* are also valid *in vivo* and to what extent this has an impact on mineral composition of the faeces.

4.3.2 Material and methods

1. Evaluation of different photometric methods for phytate analysis.

Preparation of JKM extracts

Detoxified JKM was supplied by Jatrosolutions GmbH, Hohenheim. If not stated differently, samples were treated the following way: 0.5 g of JKM was given to 10 ml of 3.5% HCl and shaken for 2 h in a magnetic stirrer at room temperature to extract all soluble anions. The sludge was centrifuged at 3 000 rpm for 10 min. An aliquot of 2 ml of the supernatant was centrifuged again at 10 000 rpm for 10 min. Subsequently, 1 ml of the supernatant (2 ml for method II) was diluted 1:5 times and this solution served as the basis for all following analysis. All extractions were made in triplicate.

Preparation of samples for different analytical methods of phytate determination

Method I: Vaintraub and Lapteva (1988)

One ml of Wade reagent (0.03% $FeCl_3 \cdot H_20$ and 0.3% sulfosalicylic acid in distilled water) was added to 0.1 ml of the sample solution and 2.9 ml of water. Samples were vortexed and centrifuged at 3000 rpm for 10 minutes.

Method II: Gao et al. (2007)

1 g of NaCl was dissolved in the sample, vortexed and left at 4°C for 60 min. This treatment was supposed to dispose of matrix components, which are thought to disturb the Wade reaction. The sample was then centrifuged at 10°C and 3 000 rpm for 20 min. 1 ml of the supernatant was diluted 1 / 25 and 3 ml was mixed with 1 ml of Wade-reagent.

Method III: Latta and Eskin (1980)

The sample solution was again diluted 1:5 times and adjusted to pH 6 with 1 M NaOH. 5 ml plastic syringes containing 0.5 g of anion exchange column (Dowex 1x8 200 - 400 (Cl)) held by a glass fiber filter were prepared. 5 ml of the sample solution were run through the column at a maximum speed of 5 ml per hour. The column was washed with 10 ml of 0.1 M NaCl solution. Phytate was subsequently extracted with 15 ml of 0.7 M NaCl solution. 3 ml of the extraction solution were mixed with 1 ml of Wade reagent and analyzed in the photometer against a standard curve.

For methods I-III, absorption was measured at 500 nm in a Helios Beta photometer (ThermoScientific) measured against a standard curve containing sodium phytate (Sigma, P-8810).

2. Identification of factors influencing the photometric determination of phytate.

Anion and IP analysis

Measurements of phytate, IP5 and IP4, phosphate, malate, citrate, sulphate and oxalate were conducted by high pressure ion chromatography (HPIC) according to Lohaus et al. (2001) at the Institute of Molecular Plant Science, Bergische University Wuppertal, Germany. As a reference material commercially available soybean meal was used. Unfortunately, no standards for IP3, IP2 and IP1 were available, so these components could not be measured.

Evaluation of potential interferences of other anions with the Wade-reagent

A series of 3 ml aqueous solutions containing 0, 20, 40, 80, 120 and 200 µg of sodium phytate, disodium oxalate, citrate monohydrate and sodiumdihydrogenphosphate were prepared and added to 1 ml of Wade- reagent. Absorptions at 500 nm were measured. A linear curve was fitted through the results.

3. *In vitro* degradation of phytate through phytase in JKM.

Three treatments each with n = 3 replicates were prepared. Treatment one ("Phytase") was 0.5 g JKM spiked with 2000 U / kg phytase (Ronozyme P (L), DSM). 0.3 ml of 2 M HCl was added to provide a pH of 4.5 and filled up to 5 ml with distilled water in a glass beaker. The beakers were sealed with parafilm and incubated in a shaking device for 24 h at 45°C. Treatment two ("Control") and three ("JKM") were the same as treatment one only without phytase addition or incubation, respectively. After sample treatment, 5 ml of 7% HCl was added to the samples and extracted for 2 hours on a magnetic stirrer. Acid extracts were centrifuged, diluted 1 / 25 and analyzed via HPIC.

4. *In vivo* phytase application on the phytate and mineral composition of faeces of Nile tilapia (*Oreochromis niloticus*) fed JKM-based diets.

Tilapia (n = 3) were selected with a starting weight around 40 g. They were held individually in 45 L aquaria attached to a recirculation system with 26°C water temperature. Two diets were prepared, each with 60.4% inclusion of JKM as the main protein source: Diet "Phytase" contained 2000 U / kg phytase (Ronozyme P (L), DSM), diet "Control" was of the same composition only without phytase (Table 4.3.1). In both diets, deficient amino acids were complemented by adding crystallized amino acids. Fish were fed daily ratios of 5 times their metabolic growth rate demonstrated by Richter et al. (2002). After one week acclimation period for the fish, faeces were collected every day until a sufficient amount for analysis was gathered. Faeces of one replicate were pooled, freeze-dried and homogenized. Phytate and other inositol phosphates of the diets and the faeces were analyzed with HPIC.

Proximate analysis of nutrients and minerals was conducted according to standard methodological procedures at the Institute of Aquaculture, Stirling, UK.

Statistical analysis

Statistical analysis was conducted with Statistica 8.0 software. Data was analyzed for statistical differences through analysis of variance (ANOVA). For the evaluation of different photometric methods for phytate analysis, Dunnett test was chosen as post-hoc test to compare treatments against the control. Student´s t-test was used to detect differences between two single samples. Percentages were arcsine transformed before analysis. Statistical significance was assumed for p-values lower than 0.05. All values in tables and graphs are shown in ± standard deviation.

4.3.3 Results

1. Evaluation of different photometric methods for phytate analysis

Figure 4.3.1 shows the results of the phytate measurement with different photometric methods. All photometric methods show significantly higher values than the control value measured with HPIC (Method I: 7.83 ± 0.44%; Method II: 7.99 ± 0.47%; Method III: 5.86 ± 0.85%; HPIC: 2.42 ± 0.38%).

Table 4.3.1 Composition of the two experimental diets with and without phytase addition fed to Nile tilapia to obtain faeces for inositol phosphate and mineral analysis.

Ingredient (%)	**Phytase**	**Control**
JKM	60.4	60.4
Wheat meal	21.7	21.7
Sunflower oil	4.5	4.5
Fish oil	5.2	5.2
Cellulose	0.4	0.4
Vitamin premix[1)]	2.0	2.0
Mineral premix[2)]	2.0	2.0
Histidine	0.1	0.1
Methionine	0.8	0.8
Lysine	2.1	2.1
Threonine	0.2	0.2
Valine	0.6	0.6
Phytase (U / kg)	2000	0
Proximate analysis (%)		
Dry matter	88.4	91.9
Crude protein	43.7	42.4
Crude lipid	11.6	10.2
Ash	9.7	10.2
Phytate[3)]	1.57	1.57
P	1.35	1.36
Ca	0.94	0.95
Mg	0.79	0.80
Zn (mg / g)	0.09	0.09

[1)] Vitamin premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg, unless otherwise stated): Vitamin A: 500 000 I.E./kg; Vitamin D3: 50 000 I.E./kg; Vitamin E: 2500; Vitamin K3 (as Menadione): 1000; Vitamin B1: 5000; Vitamin B2: 5000; Vitamin B6: 5000; Vitamin B12: 5; Nicotinic acid: 25 000; Pantothenic acid: 10000; Folic acid: 1000; Biotin: 250; Choline chloride: 100 000; Inositol: 25 000; Vitamin C: 20 125.

[2)] Mineral premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg mix): Calcium: 122 160; Phosphorus: 83 670; Magnesium: 14 960; Sodium: 18 180; Potassium: 210,250; Sulfur: 15 460; Chlorine: 29 720; Iron: 1220; Manganese: 1000; Zinc: 1630; Copper: 155; Iodine: 120.

[3)] calculated based on values analyzed by HPIC (Table 4.3.2). Values for wheat meal adapted from Storebakken et al., 2000.

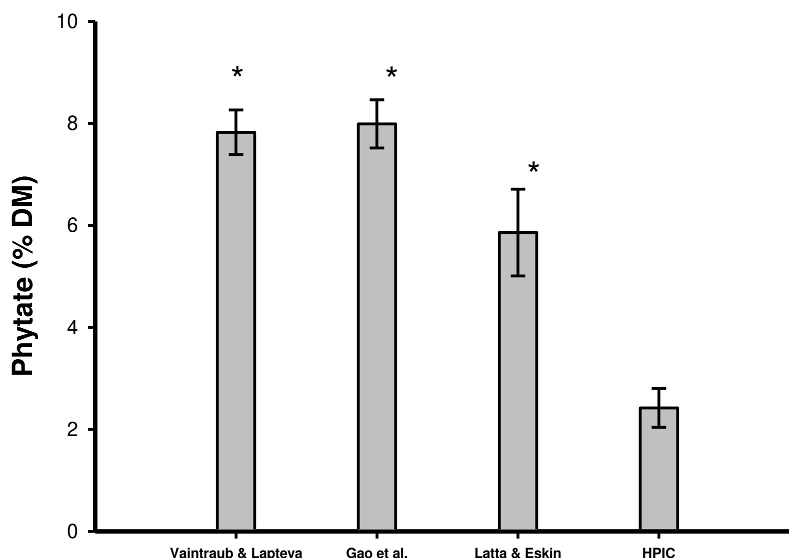


Figure 4.3.1 Phytate content of JKM measured with different colorimetric methods.
Method I (Vaintraub & Lapteva), II (Gao et al.) and III (Latta & Eskin) show significantly higher values than the control method (HPIC). Values in ± standard deviation.

Table 4.3.2 Anion content of JKM and soybean meal acid extract.

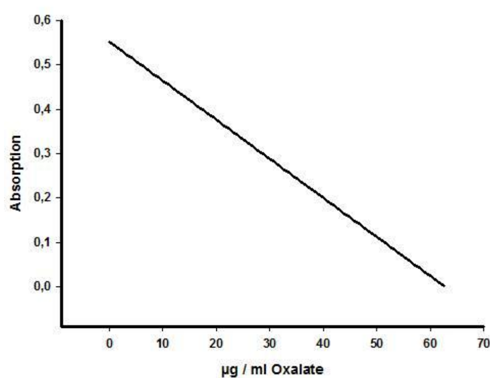
Anion	JKM	Soybean meal
Phytate	2.58	0.63
Phosphate	1.12	0.29
Citrate	1.79	2.95
Malate	0.40	0.42
Sulfate	0.46	0.40
Oxalate	2.44	0.20

Values in % DM
Samples measured with HPIC.

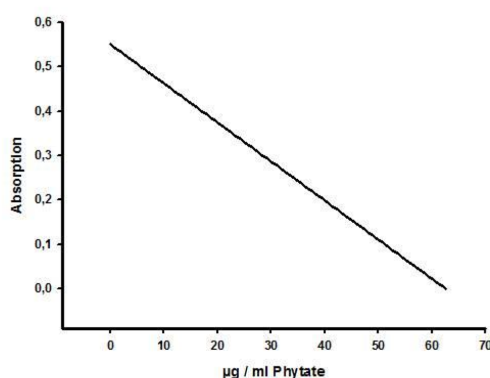
2. Identification of factors influencing the photometric determination of phytate.

The anion composition of the JKM acid extract is shown in Table 4.3.2. Phytate, phosphate and oxalate are higher than in soybean meal, while citrate is lower. The standard curves of the Wade-reagent show a significant interaction of oxalate

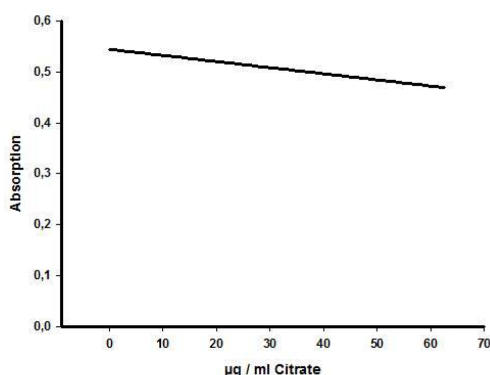
and citrate with the Wade-reagent (Figure 4.3.2). The slope of the oxalate curve is similar to the slope of phytate, while the slope of citrate is lower. Phosphate concentration had no influence on absorption of the Wade-reagent.



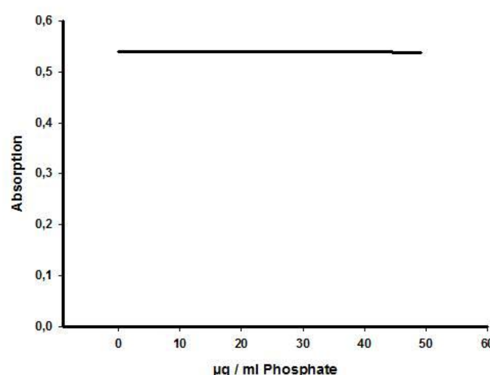
Oxalate: Slope: -0.0086, R^2 = 0.99



Phytate: Slope: -0.0088, R^2 = 0.99



Citrate: Slope: -0.0011, R^2 = 0.97



Phosphate: R^2 = 0.13

Figure 4.3.2 Interference of selected anions of JKM-extract on absorption of the Wade-reagent. From up left to down right: Phytate, oxalate, citrate, phosphate. Malate and sulfate showed no interference with the Wade reagent (not shown).

3. ***In vitro*** degradation of phytate through phytase in JKM.

In vitro results show that untreated JKM-extract contains 2.42% phytate and 0.57% IP5 (Table 4.3.3), but no IP4. Samples incubated with phytase did not contain any IPs. Incubation of JKM without phytase (control) reduced the phytate content to zero, with IP5 decreasing slightly to 0.43% and the content of IP4 increasing to 0.47%.

Available P content was increased from 0.17% in untreated samples to 0.37% in control and 1.14% in incubated samples.

Table 4.3.3 IP-values of acid extract of JKM with no incubation (“JKM”) or after *in vitro* treatment with (“Phytase”) and without phytase (“Control”).

Treatment	IP6	IP5	IP4	IP-P[1)]	Available P[2)]	IP-P + Available P
JKM	2.42	0.57	-	0.84	0.17	1.01
Phytase[3)]	-	-	-	-	1.14	1.14
Control	-	0.43	0.47	0.24	0.37	0.61

Values in %

[1)] IP-P determined with following formula: IP-P (%) = IP6 (%) * 0.282 + IP5 (%) * 0.274 + IP4 (%) * 0.264

[2)] Available P measured as inorganic phosphate (PO_4^{3-}) in the JKM-extract

[3)] 2000 U / kg

4. Effect of phytase application on the phytate and mineral composition of faeces of Nile tilapia (*Oreochromis niloticus*) fed JKM-based diets.

Table 4.3.4 shows the composition of phytate, IP5 and IP4 in faeces of fish fed the two different experimental diets to Nile tilapia (*Oreochromis niloticus*). Fish fed the phytase containing diet had significantly lower IP6, IP5 and IP4 values than control fish. This was reflected in lower content of minerals in the faeces, with significantly lower values observed for phosphorus, calcium and zinc, while magnesium was not significantly different to the control (Table 4.3.5).

Table 4.3.4 IP-concentration in faeces of Nile tilapia fed diets with (“Phytase”) and without phytase (“Control”).

	IP6		IP5		IP4	
Control	12.0 ± 0.79	p = 0.001	4.60 ± 0.07	p = 0.003	0.17 ± 0.06	p = 0.009
Phytase	5.27 ± 0.80		3.18 ± 0.37		0.00 ± 0.00	

Values in µmol / g

p-values ≤ 0.05 indicate statistically significant differences between samples.

Table 4.3.5 Faeces mineral analysis of Nile tilapia fed JKM-based diets with ("Phytase") and without phytase ("Control").

	P (%)		Ca (%)		Mg (%)		Zn (‰)	
Control	3.61 ± 0.32	p = 0.050	7.14 ± 0.29	p = 0.048	0.66 ± 0.14	p = 0.570	0.78 ± 0.09	p = 0.044
Phytase	2.92 ± 0.29		6.50 ± 0.27		0.61 ± 0.03		0.63 ± 0.03	

p-values ≤ 0.05 indicate statistically significant differences between samples.

4.3.4 Discussion

1. Evaluation of different photometric methods for phytate analysis

The amount of phytate in *Jatropha curcas* kernel meal was determined using three photometric methods and those were compared to a reference method using HPIC. The photometric methods originated from Vaintraub and Lapteva (1988), Latta and Eskin (1980) and Gao et al. (2007). It was shown that the photometric methods overestimate the true phytate content by 400% (Vaintraub & Lapteva, Gao et al.) and 250% (Latta & Eskin) respectively. It can therefore be said that these photometric methods are not adequate to determine phytate in JKM. Previous results as referred to in the introduction therefore have to be corrected for the true phytate content. Overestimation of phytate content with the present photometric methods have been documented before; however, the problems were associated with lower IPs (IP5 – IP1) interfering with the correct analysis (Lehrfeld & Morris, 1992; Rounds & Nielsen, 1993). Lower IPs do not have the negative effect on nutrient absorption as does IP6 (Lönnerdal et al., 1989) and therefore need to be left out from the measurement if an accurate impact of phytate content on nutrient availability is to be assessed. Naturally 97% of all IPs are present in form of IP6 (Kasim & Edwards, 1998) and only a small percentage as IP5. Processed plant meals have higher percentages of IP5, but contain no IP4 or lower IPs (Kasim & Edwards, 1998). This was confirmed by our measurements of JKM-extract (Table 4.3.3), which contained 2.42% of IP6 and 0.57% of IP5 and no lower IPs.

2. Identification of factors influencing the photometric determination of phytate.

We could show that the reason for the overestimation of phytate when measuring with methods involving the Wade-reagent lies in other anions present in the JKM-extract, predominantly oxalate. The equation of oxalate decoloration of the color-complex of the Wade reagent shows the same slope as does phytate. The interference of citrate with the Wade reagent is less pronounced. It is known that iron (Fe^{2+}) forms insoluble complexes with oxalate in solutions (Noonan & Savage, 1999; Savage et al., 2000). The magnitude of the error of a phytate determination involving the Wade-reagent therefore depends on the concentration of these compounds in the respective plant meal. For example, soybean meal only contains about 10% of oxalate compared to JKM and therefore the error deriving from this substance will be less for this meal. Citrate content is relatively equal in both meals, but due to its lower affiliation to Fe^{2+} has a smaller effect on measurement accuracy. Unlike Vaintraub & Lapteva (1988), we found no influence of phosphate on the decoloration of the Wade reagent. It may be concluded that despite their practicability the photometric methods involving the Wade reagent are prone to errors because of other anions binding to Fe^{2+} of the Wade reagent and therefore this method should only be applied if the concentration and their degree of influence on the analysis is known.

3. *In vitro* degradation of phytate through phytase in JKM.

JKM was incubated in a phytase (2000 U / kg) solution to determine to what extent phytase pretreatment would reduce phytate content. After 24 h incubation, there was no phytate or other IPs left in the samples. Nwanna et al. (2007) pre-incubated a diet made of various plant-based feedstuffs at 40°C for 15.5h and found phytate to be reduced from 0.41% to 0.02% while an incubated non-phytase control reduced phytate content only to 0.25%. In the present experiment, even the non-phytase

treated control diet had no phytate left; however, there were considerate amounts of IP5 and IP4 left in the sample.

IP-P plus available P is considered to equal total P concentration and should be similar in all of the present treatments (Table 4.3.3). While some variability may be attributed to the analytic procedure ("Phytase" (1.14%) and "JKM" (1.01%), treatment "Control" showed much lower total P (0.61%). The amount of available P in this treatment only slightly increased due to incubation (0.37%) compared to the untreated treatment "JKM" (0.17%). It is likely that the difference in total available phosphorus is due to incomplete hydrolysis of IP6 and IP5, with high concentrations of IP1 – IP3 remaining in treatment "Control".

Pre-incubation of JKM with phytase completely erases all IPs from the sample. Phytase pre-incubation of JKM may therefore be an efficient step to maximize P-availability to fish and minimize P output in the effluent water.

4. Effect of phytase application on the phytate and mineral composition of faeces of Nile tilapia (*Oreochromis niloticus*) fed JKM-based diets.

Tilapia were fed a JKM-based diet containing phytase (2000 U / kg). Phytate, IP5 and IP4 content of faeces was measured and compared to faeces from fish fed a control diet without phytase. Unlike *in vitro* pre-incubation of JKM with phytase, it was shown that phytate in faeces was reduced only about 56% *in vivo*. Still, mineral faeces values were lower for phytase-containing diets, suggesting a better availability of minerals for fish fed this diet. The improvement of phytase on mineral availability for fish has been known for a while (Cao et al., 2007). In the present trial, however, it is interesting to see that a large fraction of phytate remains in the faeces despite phytase addition. Therefore, it may be cost-efficient to pre-incubate JKM to maximize available phosphate and other minerals and minimize the need to supply minerals in form of premixes.

Conclusion

The present trial clarifies false measurements previously conducted for phytate in JKM and documents phytase application *in vitro* and *in vivo*. Future work could involve a comparison between JKM-based phytase-containing feed with and without pre-incubation to quantify the potential additional value of the pre-incubation step for tilapia.

4.3.5 References

Aderibigbe, A. O., Johnson, C. O. L. E., Makkar, H. P. S., Becker, K., & Foidl, N. (1997). Chemical composition and effect of heat on organic matter- and nitrogen-degradability and some antinutritional components of *Jatropha* meal. *Animal Feed Science and Technology*, *67*(2-3), 223–243.

Akinleye, A. O., Kumar, V., Makkar, H. P. S., Angulo-Escalante, M. A., & Becker, K. (2011). *Jatropha platyphylla* kernel meal as feed ingredient for Nile tilapia (*Oreochromis niloticus* L.): growth, nutrient utilization and blood parameters. *Journal of Animal Physiology and Animal nutrition*, *96*(1), 1–11.

Cao, L., Wang, W., Yang, C., Yang, Y., Diana, J., Yakupitiyage, A., … Li, D. (2007). Application of microbial phytase in fish feed. *Enzyme and Microbial Technology*, *40*(4), 497–507.

Devappa, R. K., Makkar, H. P. S., & Becker, K. (2012). Localisation of antinutrients and qualitative identification of toxic components in *Jatropha curcas* seed. *Journal of the Science of Food and Agriculture*, *92*(7), 1519–1525.

Gao, Y., Shang, C., Saghai Maroof, M. A., Biyashev, R. M., Grabau, E. A., Kwanyuen, P., … Buss, G. R. (2007). A modified colorimetric method for phytic acid analysis in soybean. *Crop Science*, *47*(5), 1798 – 1803.

Kasim, A. B., & Edwards, H. M. (1998). The analysis for inositol phosphate forms in feed ingredients. *Journal of the Science of Food and Agriculture*, *76*(1), 1–9.

Kumar, V., Makkar, H. P. S., & Becker, K. (2010). Dietary inclusion of detoxified *Jatropha curcas* kernel meal: effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L. *Fish Physiology and Biochemistry*, *36*(4), 1159–1170.

Kumar, V., Makkar, H. P. S., & Becker, K. (2012a). Evaluations of the nutritional value of *Jatropha curcas* protein isolate in common carp (*Cyprinus carpio* L.). *Journal of Animal Physiology and Animal Nutrition*, *96*(6), 1030–1043.

Kumar, V., Sinha, A. K., Makkar, H. P. S., De Boeck, G., & Becker, K. (2012b). Phytate and phytase in fish nutrition. *Journal of Animal Physiology and Animal Nutrition*, *96*(3), 335–364.

Latta, M; Eskin, M. (1980). A simple and rapid colorimetric method for phytate determination. *J. Agric. Food Chem*, *28*(6), 1313–1315.

Lehrfeld, J., & Morris, E. R. (1992). Overestimation of phytic acid in foods by the AOAC Anion-Exchange method. *J. Agric. Food Chem.*, *40*(11), 2208–2210.

Lohaus, G., Pennewiss, K., Sattelmacher, B., Hussmann, M., & Hermann Muehling, K. (2001). Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. *Physiologia Plantarum*, *111*(4), 457–465.

Lönnerdal, B., Sandberg, A., Sandström, B., & Kunz, C. (1989). Inhibitory effects of phytic acid and other inositol phosphates on zinc and calcium absorption in suckling rats. *The Journal of Nutrition*, *119*(2), 211–214.

Makkar, H. P. S., Becker, K., Sporer, F., & Wink, M. (1997). Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. *J. Agric. Food Chem.*, *45*(8), 3152–3157.

Makkar, H. P. S., & Becker, K. (2009). *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. *European Journal of Lipid Science and Technology*, *111*(8), 773–787.

Martinez-Herrera, J., Siddhuraju, P., Francis, G., Davilaortiz, G., & Becker, K. (2006). Chemical composition, toxic/antimetabolic constituents, and effects of different treat-

ments on their levels, in four provenances of *Jatropha curcas* L. from Mexico. *Food Chemistry*, *96*(1), 80–89.

Noonan, S. C., & Savage, G. P. (1999). Oxalate content of foods and its effect on humans. *Asia Pacific J Clin Nutr*, *8*(1), 64–74.

Nwanna, L., Eisenreich, R., & Schwarz, F. (2007). Effect of wet-incubation of dietary plant feedstuffs with phytases on growth and mineral digestibility by common carp (*Cyprinus carpio* L). *Aquaculture*, *271*(1-4), 461–468.

Pradhan, S., Naik, S. N., Khan, M. A. I., & Sahoo, P. (2011). Experimental assessment of toxic phytochemicals in Jatropha curcas: oil, cake, bio-diesel and glycerol. *Journal of the Science of Food and Agriculture*, *92*(3), 511–519.

Harter, T., Buhrke, F., Kumar, V., Focken, U., Makkar, H. P. S., & Becker, K. (2011). Substitution of fish meal by *Jatropha curcas* kernel meal: Effects on growth performance and body composition of white leg shrimp (*Litopenaeus vannamei*). *Aquaculture Nutrition*, *17*(5), 542–548.

Ravindran, V., Ravindran, G., & Sivalogan, S. (1994). Total and phytate phosphorus contents of various foods and feedstuffs of plant origin. *Food Chemistry*, *50*(2), 133–136.

Richter, H., Francis, G., & Becker, K. (2002). A reassessment of the maintenance ration of red tilapia. *Aquaculture International*, *10*(1), 1–9.

Rounds, M. A., & Nielsen, S. S. (1993). Anion-exchange high-performance liquid chromatography with post-column detection for the analysis of phytic acid and other inositol phosphates. *Journal of Chromatography A*, *653*(1), 148–152.

Saetae, D., & Suntornsuk, W. (2010). Toxic compound, anti-nutritional factors and functional properties of protein isolated from detoxified *Jatropha curcas* seed cake. *International Journal of Molecular Sciences*, *12*(1), 66–77.

Savage, G. P., Vanhanen, L., Mason, S. M., & Ross, A. B. (2000). Effect of cooking on the soluble and insoluble oxalate content of some New Zealand foods. *Journal of Food Composition and Analysis*, *13*(3), 201–206.

Storebakken, T., Shearer, K. D., & Baeverfjord, G. (2000). Digestibility of macronutrients, energy and amino acids, absorption of elements and absence of intestinal enteritis in Atlantic salmon, *Salmo salar*, fed diets with wheat gluten. *Aquaculture*, *184*(1-2), 115–132.

Vaintraub, I. A., & Lapteva, N. A. (1988). Colorimetric determination of phytate in unpurified extracts of seeds and the products of their processing. *Analytical Biochemistry*, *175*(1), 227–230.

Wade, H.E.; Morgan, D. M. (1953). Detection of phosphate esters on paper chromatograms. *Nature*, *171*, 529–530.

Xiao, J., Zhang, H., Niu, L., Wang, X., & Lu, X. (2011). Evaluation of detoxification methods on toxic and antinutritional composition and nutritional quality of proteins in *Jatropha curcas* meal. *Journal of Agricultural and Food Chemistry*, *59*(8), 4040–4044.

5 Dietary Oxalate as an Anti-nutritional Factor in Basic Diets for Carp and Tilapia

5.1 Effect of dietary sodium-oxalate on growth, body composition, nutrient retention and blood parameters of common carp (*Cyprinus carpio*)

September 2013 – January 2014

Carsten Krome[1,2], Getrud Lohaus[3], Kim Jauncey[2], Ulfert Focken[1]

[1)] Thuenen Institute of Fisheries Ecology, Ahrensburg, Germany

[2)] Institute of Aquaculture, University of Stirling, UK

[3)] Institute of Molecular Plant Science, Bergische University Wuppertal, Germany

Abstract

Common carp (*Cyprinus carpio*) were fed diets containing oxalate, a potentially anti-nutritive ingredient of many plant feedstuffs, at levels of 0%, 0.5%, 1.5% and 2.5% for 8 weeks. Fish fed diets containing 1.5% and 2.5% oxalate showed significantly higher specific growth rate than those fed diets containing 0% or 0.5% (2.09 - 2.11% and 1.74 - 1.88%, respectively, $p \leq 0.05$). Body composition was also significantly influenced by dietary oxalate concentration with fish fed diets 1.5% and 2.5% showing significantly lower body lipid and higher ash content (lipid: 5.29 – 5.67% compared to 7.13 – 7.27%; ash: 2.65 – 2.74% compared to 2.38%, wet weight basis, $p \leq 0.05$). Body mineral analysis showed higher calcium, magnesium and phosphorus retention in diets 1.5% and 2.5%. Blood plasma analysis showed no significant differences between treatments, however, a tendency towards higher cholesterol and glucose levels in plasma of fish fed diets 0% and 0.5% could be shown. In conclusion, the expected anti-nutritional effects of oxalate on mineral availability were not

observed; on the contrary, oxalate seemed to have positive effects on growth and mineral retention.

5.1.1 Introduction

In this trial, the effect of four different concentrations (0%, 0.5%, 1.5% and 2.5%) of soluble oxalate in a standard diet on the growth performance, body composition and mineral availability of common carp (*Cyprinus carpio*) were investigated.

5.1.2 Material and methods

Diet formulation

Four isonitrogeneous and isoenergetic diets were produced containing 0% (Control), 0.5%, 1.5% and 2.5% of di-sodium-oxalate (Table 5.1.1). Protein content was formulated to be 32% and lipid content 12% (50% fish oil, 50% sunflower oil). Vitamins and minerals were supplied in the form of a premix. Essential amino acids were added to meet the requirements of carp (NRC, 2011). Dietary ingredients were mixed with a standard kitchen blender. Di-sodium- oxalate was slowly sieved into the mix to ensure an even distribution. 40% water was added and the mix was passed through a meat mincer to form noodles, which were then crumbled and air-dried at 45° for 48 hours.

Table 5.1.1 Composition of experimental diets containing 0%, 0.5%, 1.5% and 2.5% of oxalate fed to common carp for 8 weeks.

Ingredient (g / kg)	Control	0.5%	1.5%	2.5%
Fishmeal	388.0	388.0	388.0	388.0
Wheat meal	441.5	441.5	441.5	441.5
Sunflower oil	52.0	52.0	52.0	52.0
Fish oil	22.0	22.0	22.0	22.0
Cellulose	37.5	30.0	15.0	0.0
Vitamin premix[1)]	20.0	20.0	20.0	20.0
Mineral premix[2)]	20.0	20.0	20.0	20.0
NaH_2PO_4	10.0	10.0	10.0	10.0
Na_2-oxalate	0.0	7.5	22.5	37.5
Threonine	4.4	4.4	4.4	4.4
Phenylalanine	1.8	1.8	1.8	1.8
Lysine	3.3	3.3	3.3	3.3
Proximate composition				
Dry matter	923.7	925.5	933.2	922.2
Crude protein	335.7	328.7	322.3	321.5
Crude lipid	124.0	126.4	122.1	124.4
Crude ash	101.3	104.0	112.3	120.6
Oxalate	0.33	3.34	11.2	21.3
Gross energy (kJ / g)	19.3	19.0	18.8	18.4
Digestible energy (kJ / g)[3)]	14.9	14.9	14.9	14.9
Minerals				
Na (g / kg)	7.28	9.82	15.1	20.2
Mg (g / kg)	2.22	2.14	2.08	1.93
P (g / kg)	16.0	15.2	14.5	14.1
K (g / kg)	6.09	6.04	5.93	5.90
Ca (g / kg)	24.8	23.5	22.7	21.2
Fe (g / kg)	0.25	0.24	0.22	0.25
Mn (mg / kg)	57.3	55.4	55.1	56.1
Zn (mg / kg)	0.12	0.11	0.11	0.10
Co (mg / kg)	0.67	0.57	0.64	0.62
Cu (mg / kg)	9.60	19.0	11.1	9.68

Values based on wet weight

[1)]Vitamin premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg, unless otherwise stated): Vitamin A: 500,000 I.E./kg; Vitamin D3: 50,000 I.E./kg; Vitamin E:2500; Vitamin K3 (as Menadione): 1000; Vitamin B1: 5000; Vitamin B2:5000; Vitamin B6: 5000; Vitamin B12: 5; Nicotinic acid: 25000; Pantothenic acid: 10000; Folic acid: 1000; Biotin: 250; Choline chloride: 100000; Inositol: 25000; Vitamin C: 20125.

[2)] Mineral premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg mix): Calcium: 122,160; Phosphorus: 83,670; Magnesium: 14,960; Sodium: 18,180; Potassium: 210,250; Sulfur: 15,460; Chlorine: 29,720; Iron: 1220; Manganese: 1000; Zinc: 1630; Copper: 155; Iodine: 120.

[3)] calculated using available energy constants for carbohydrates (12.6 kJ / g), proteins (19.6 kJ / g) and lipids (33.5 kJ / g).

Experimental animals and setup

Cyprinus carpio were hatched and reared to the initial experimental weight of 9.85 ± 0.23 g at the Thuenen Institute of Fisheries Ecology, Ahrensburg, Germany. A total of 100 experimental fish (five replicates for each treatment, each replicate containing 5 fish) were distributed into a total of twenty 40 L-aquarium tanks connected to a recirculation system. Water temperature was 26°C, with 1.5 L / min/tank flow rate. Fish were acclimatized to the system for one week. The experimental duration was 8 weeks. Water quality parameters were measured on a weekly basis and were within the following ranges throughout the experiment (except for pH, values in mg / l): pH: 6.5 – 6.7; O_2: 7.57 – 8.79; NH_4^+: 0.02 – 0.055; NO_2^-: 0.05 – 0.07; NO_3^-: 880 – 950. Faeces were collected daily throughout the last two weeks of the experiment by stripping the hindgut of the fish.

As a parameter for feed quantity, the metabolic maintenance ration of the fish was calculated (see 2.1.3).

Pre- and post experimental procedures, chemical analyses as well as performance calculations and and equations were conducted as outlined in Chapter 2.

Statistics

Data was tested for normal distribution with the Shapiro-Wilk test. Percentages were arcsine transformed before analysis. One-way ANOVA ($p \leq 0.05$) was used to analyze treatments. Tukey's test was applied as post-hoc test. Statistics were conducted with Statistica 8 software. Values are expressed as mean ± standard deviation.

Table 5.1.2 Body mass gain of common carp fed different concentrations of soluble oxalate for 8 weeks.

Treatment	Control				0.5%				1.5%				2.5%			
IW (g)	9.87	±	0.20		9.90	±	0.29		9.83	±	0.27		9.81	±	0.23	
FW (g)	26.2	±	1.09	b	28.4	±	2.61	a,b	31.5	±	2.23	a	32.1	±	2.32	a
BMG (g)	16.3	±	1.26	b	18.5	±	2.48	a,b	21.6	±	2.01	a	22.3	±	2.28	a
BMG (%)	165.1	±	15.9	c	186.9	±	23.2	b,c	219.8	±	16.0	a,b	225.9	±	23.4	a

Values are mean +/- standard deviation, n = 5. Values with different superscripts are significantly different from each other ($p \leq 0.05$). IW: Initial weight; FW: Final weight; BMG: body mass gain.

5.1.3 Results

All diets were consumed at all times and fish survival was 100%. Body mass gain of fish was higher in all diets containing oxalate than in the control (0%) diet, with differences between treatments 1.5% and 2.5% being significant ($p \leq 0.05$, Table 5.1.2). The same was true for SGR, MGR and PER (Table 5.1.3). FCR was significantly lower for treatments 1.5% and 2.5% than the control diet, while treatment 0.5% was not. PPV was higher for treatments 1.5% and 2.5% than for lower oxalate treatments. LPV was significantly higher in the control diet than in diets 1.5% and 2.5%, while there was no difference in EPV (Table 5.1.3). There were significant differences in the body composition of experimental fish, the control treatment and treatment 0.5% being significantly higher than 1.5% and 2.5% in body lipid and energy and significantly lower in body moisture and ash contents (Table 5.1.4). There was no difference in body protein content. Body mineral analysis showed the control and 0.5% treatment to be significantly lower in sodium, magnesium, phosphorus and calcium, but significantly higher in body zinc content (Table 5.1.4). Mineral analysis of the faeces showed opposite trends, except for zinc content, which was also higher in treatments 0% and 0.5% (Table 5.1.5). Calcium, magnesium and phosphorus retention was significantly lower in low oxalate treatments (0% and 0.5%) compared to higher oxalate treatments (1.5% and 2.5%). There was no difference between blood plasma calcium among treatments and no

significant differences could be found between other blood parameters, though plasma cholesterol and glucose tended to be higher in the control and the 0.5% treatment than in treatment 1.5% and 2.5% (Table 5.1.7).

Table 5.1.3 Nutrient utilization of common carp fed different concentrations of oxalate for 8 weeks.

Treatment	Control	0.5%	1.5%	2.5%
SGR	1.74 ± 0.11 ^{b}	1.88 ± 0.15 ^{a,b}	2.07 ± 0.09 ^{a}	2.11 ± 0.13 ^{a}
FCR	1.74 ± 0.10 ^{a}	1.61 ± 0.15 ^{a,b}	1.45 ± 0.07 ^{b}	1.47 ± 0.11 ^{b}
MGR	7.35 ± 0.43 ^{b}	7.96 ± 0.65 ^{a,b}	8.80 ± 0.42 ^{a}	8.95 ± 0.54 ^{a}
PER	1.72 ± 0.11 ^{a}	1.90 ± 0.18 ^{a,b}	2.14 ± 0.11 ^{b}	2.12 ± 0.16 ^{b}
PPV	22.9 ± 1.34 ^{b}	24.8 ± 2.51 ^{b}	28.2 ± 1.51 ^{a}	28.2 ± 2.64 ^{a}
LPV	43.5 ± 1.14 ^{a}	44.0 ± 6.26 ^{a}	36.8 ± 5.66 ^{a,b}	33.4 ± 2.71 ^{b}
EPV	20.6 ± 0.53	21.4 ± 2.23	21.0 ± 1.55	20.8 ± 1.55

Values are mean +/- standard deviation, n = 5. SGR: specific growth rate (% / day); FCR: feed conversion ratio; MGR: metabolic growth rate (g * $kg^{0.8}$ / day); PER: protein efficiency ratio; PPV: protein productive value (%); LPV: lipid productive value (%); EPV: energy productive value (%). Values with different superscripts are significantly different from each other ($p \leq 0.05$)

Table 5.1.4 Body nutrient and mineral composition of common carp fed different concentrations of oxalate for 8 weeks.

Treatment	Initial Fish	Control	0.5%	1.5%	2.5%
Moisture (%)	77.5 ± 0.67	75.1 ± 0.53 ^{b}	75.6 ± 0.34 ^{b}	76.8 ± 0.74 ^{a}	77.0 ± 0.59 ^{a}
Crude protein (%)	14.6 ± 0.33	13.80 ± 0.14	13.6 ± 0.16	13.6 ± 0.21	13.7 ± 0.31
Crude lipid (%)	3.82 ± 0.38	7.27 ± 0.36 ^{a}	7.13 ± 0.48 ^{a}	5.67 ± 0.67 ^{b}	5.29 ± 0.27 ^{b}
Ash (%)	3.35 ± 0.18	2.38 ± 0.08 ^{b}	2.38 ± 0.07 ^{b}	2.65 ± 0.09 ^{a}	2.74 ± 0.09 ^{a}
Gross energy (kJ/g)	4.89 ± 0.27	6.13 ± 0.17 ^{a}	5.94 ± 0.12 ^{a}	5.44 ± 0.25 ^{b}	5.31 ± 0.16 ^{b}
Minerals					
Na (g / kg)	1.14 ± 0.06	0.82 ± 0.04 ^{b}	0.80 ± 0.05 ^{b}	0.95 ± 0.08 ^{a}	0.93 ± 0.04 ^{a}
Mg (g / kg)	0.31 ± 0.01	0.27 ± 0.00 ^{b}	0.25 ± 0.01 ^{b}	0.28 ± 0.01 ^{a}	0.29 ± 0.01 ^{a}
P (g / kg)	5.25 ± 0.24	4.23 ± 0.05 ^{b}	4.11 ± 0.23 ^{b}	4.69 ± 0.26 ^{a}	4.79 ± 0.18 ^{a}
K (g / kg)	2.91 ± 0.14	2.72 ± 0.09	2.67 ± 0.14	2.80 ± 0.16	2.77 ± 0.13
Ca (g / kg)	7.15 ± 0.30	5.38 ± 0.08 ^{b}	5.23 ± 0.26 ^{b}	6.28 ± 0.33 ^{a}	6.50 ± 0.33 ^{a}
Zn (g / kg)	0.13 ± 0.01	0.09 ± 0.00 ^{a}	0.08 ± 0.01 ^{a,b}	0.07 ± 0.01 ^{b}	0.07 ± 0.00 ^{b}
Mn (mg / kg)	1.11 ± 0.12	0.69 ± 0.10	0.65 ± 0.11	0.57 ± 0.06	0.53 ± 0.07
Fe (mg / kg)	19.6 ± 1.21	10.4 ± 0.50	10.2 ± 1.25	10.5 ± 1.07	9.53 ± 0.83
Co (mg / kg)	0.009 ± 0.00	0.012 ± 0.00	0.011 ± 0.00	0.008 ± 0.01	0.011 ± 0.00
Cu (mg / kg)	1.11 ± 0.19	1.18 ± 0.10	1.14 ± 0.13	1.22 ± 0.06	0.93 ± 0.53

Values based in wet weight. Values are mean +/- standard deviation, n = 5. Values with different superscripts are significantly different from each other ($p \leq 0.05$)

Table 5.1.5 Faeces mineral composition of common carp fed different concentrations of oxalate for 8 weeks.

Treatment	0%	0.50%	1.50%	2.50%
Na (g / kg)	8.87 ± 1.42 ^{b}	10.2 ± 1.45 ^{b}	21.8 ± 1.77 ^{a}	19.2 ± 1.88 ^{a}
Mg (g / kg)	3.72 ± 0.30 ^{a,b}	3.91 ± 0.70 ^{a}	2.37 ± 0.46 ^{c}	2.88 ± 0.37 ^{b,c}
P (g / kg)	37.6 ± 1.47 ^{a}	34.2 ± 5.03 ^{a}	19.3 ± 5.46 ^{b}	22.1 ± 5.49 ^{b}
K (g /kg)	2.26 ± 0.32	2.58 ± 0.65	3.72 ± 1.58	2.80 ± 0.48
Ca (g / kg)	91.1 ± 4.86 ^{a}	89.4 ± 9.10 ^{a}	53.3 ± 14.3 ^{b}	73.0 ± 14.4 ^{a,b}
Zn (g / kg)	0.27 ± 0.07 ^{a}	0.22 ± 0.04 ^{a,b}	0.17 ± 0.04 ^{b}	0.19 ± 0.01 ^{a,b}
Mn (mg / kg)	192.0 ± 25.7	180.0 ± 41.3	152.5 ± 84.7	150.5 ± 30.8
Fe (mg / kg)	800.1 ± 129.8	734.6 ± 308.3	696.3 ± 617.5	640.9 ± 314.2
Co (mg / kg)	1.25 ± 0.13 ^{c}	1.45 ± 0.09 ^{b,c}	2.25 ± 0.25 ^{a}	2.00 ± 0.58 ^{a,b}
Cu (mg / kg)	62.7 ± 20.3	63.2 ± 8.94	88.9 ± 36.2	62.1 ± 7.48

Values are mean +/- standard deviation, n = 5. Values with different superscripts are significantly different from each other ($p \leq 0.05$)

5.1.4 Discussion

To our knowledge the present study is the first to deal with the influence of oxalate as an anti-nutritional factor in carp. Di-sodium oxalate was chosen because of its high solubility at neutral pH values compared to calcium or magnesium oxalate. The maximum treatment level of 2.5% was chosen to represent a theoretical maximal oxalate content in a fish diet consisting largely of high-oxalate plant ingredients, such as detoxified *Jatropha curcas* kernel meal. Oxalate values measured in the diets were 20-25% less than expected (Table 5.1.1). The present study shows significant impacts of oxalate on growth development, body nutrient and mineral composition of the experimental fish. There was a tendency for treatments 0% and 0.5% to show similar results, while diets 1.5% and 2.5% were different from the lower oxalate treatments, but similar to each other.

Table 5.1.6 Mineral retention of selected minerals of common carp fed different concentration of oxalate for 8 weeks.

Treatment	0%				0.50%				1.50%				2.50%			
Mg	6.15	±	0.53	a	6.51	±	0.91	a	9.22	±	0.45	b	9.88	±	0.37	b
P	12.7	±	1.01	a	14.1	±	2.08	a	21.4	±	2.26	b	22.6	±	0.82	b
Ca	9.83	±	0.99	a	11.0	±	1.82	a	18.2	±	1.98	b	20.2	±	0.64	b
Zn	30.8	±	5.10		28.7	±	6.74		31.5	±	5.47		29.3	±	2.92	

Values in %. Values are mean +/- standard deviation, n = 5. Values with different superscripts are significantly different from each other ($p \leq 0.05$)

Growth development

There was a significantly positive effect of oxalate on body mass gain, specific growth rate and feed conversion ratio of experimental fish. This effect was unexpected as a decrease in performance, possibly through mineral deficiencies or direct oxalate poisoning of fish in the high-oxalate treatments, might have been expected.

Table 5.1.7 Selected blood plasma parameters of common carp fed different level of oxalate for 8 weeks.

Treatment	0%			0.5%			1.5%			2.5%		
Ca (mg /dl)	10.9	±	0.26	11.3	±	0.43	11.4	±	0.33	11.0	±	0.49
Na (mmol / l)	125.5	±	4.51	126.4	±	4.34	130.3	±	0.96	132.6	±	3.78
K (mmol / l)	3.85	±	1.18	3.88	±	0.49	3.25	±	0.77	4.28	±	0.79
Cl (mmol / l)	134.0	±	3.27	130.6	±	5.55	134.0	±	7.79	136.8	±	7.73
Mg (mg / dl)	2.52	±	0.26	2.42	±	0.40	2.32	±	0.29	2.48	±	0.29
PO_4^- (mg / dl)	9.02	±	1.25	8.68	±	1.48	8.88	±	1.46	9.25	±	1.59
Creatinine (mg / dl)	0.42	±	0.08	0.52	±	0.13	0.46	±	0.09	0.42	±	0.04
Cholesterol (mg / dl)	193.2	±	18.7	191.0	±	35.0	163.2	±	19.6	160.0	±	2.55
Glucose (mg / dl)	128.0	±	23.7	117.2	±	29.1	90.0	±	13.0	105.6	±	14.2

Values are mean +/- standard deviation, n = 5

Oxalate is the salt of oxalic acid, a strong, organic acid (Franceschi & Horner, 1980). Organic acids or their salts have received considerable attention as growth promoting agents (Lueckstädt et al., 2008). Jongbloed et al. (2000) showed that dietary lactic acid or formic acid supplementation lead to improved weight gain and feed

conversion ratio in pigs. Gislason et al. (1996), showed dietary sodium lactate at 1.5% to increase growth rate in Arctic Char. Similarly, also in Arctic Char, specific growth rate increased from 0.61% to 0.83% and from 0.51% to 0.70% when fish were fed a diet containing 1% sodium lactate or sodium acetate, respectively (Ringo, 1991; Ringo, 1992, cited from Lueckstädt, 2008). Baruah et al. (2007) demonstrated improved growth rates for Indian carp (*Labeo rohita*) fed a diet containing 3% citric acid. In this latter study, phosphorus digestibility improved while dry matter and protein digestibility showed no significant difference to the control. The mechanisms behind improved growth rates through dietary supplements of organic acids or their salts are unclear. One possibility is improved mineral, especially phosphorus, digestibility as seen for rainbow trout fed 1% citric acid supplemented diets (Hernandez et al., 2012). In the present trial, we observed improved phosphorus retention in the high-oxalate diets (diets 1.5% and 2.5%), which were also the treatments exhibiting improved growth. However, diets were formulated to meet or exceed available phosphorus and other mineral requirements; therefore it is unlikely that higher phosphorus retention in these diets was responsible for improved growth. Another proposed mechanism of the positive effects of dietary organic acids is their antimicrobial effects leading to lower levels of pathogenic and non-pathogenic bacteria in the intestine, thereby decreasing inflammatory processes in the intestinal mucosa (Ganguly et al., 2013). This leads to higher villus length and consequently a larger surface for nutrient absorption (Ganguly et al., 2013). A possible hypothesis for the improved growth observed in the present trials may therefore be an antimicrobial function of oxalate linked to higher villi height in the intestine. Further histological and microbiological studies are required to investigate this hypothesis.

Body nutrient composition

There were significant differences in body composition of experimental fish for treatments 0% and 0.5% compared to 1.5% and 2.5%. Focken & Becker (1991), reviewed body composition across 702 different data sets for carp and found average crude lipid values of 6.46% – 7.34% (based on fresh matter) for dry weights between 74% - 75%, which is within the scope of treatments 0% and 0.5% in this trial and 5.31% lipid for fish with moisture content of around 77% valid for treatments 1.5% and 2.5%. Higher ash values in treatments 1.5% and 2.5% reflect improved mineral retention of these treatments (as discussed below). There was no impact of oxalate concentration on body protein content.

Mineral composition of carcass and faeces, mineral retention

Values for mineral composition of carcass and faeces show a clear correlation. Those minerals with higher content in carcass showed lower values in faeces and vice versa. This trend was true for calcium, magnesium and phosphorus. Sodium content in the diet increased with higher sodium oxalate content of the diet, which was reflected in higher body sodium content as well as higher faecal sodium content.

Most existing literature regarding the effects of oxalate in the digestive tract of humans or livestock, states that oxalate binds to divalent cations making these unavailable for absorption. This is shown by increased cation content in the faeces and concomitant lower digestibility and retention values for these cations (Noonan & Savage, 1999; Rahman et al., 2013). However, our results show the opposite trend with treatments 1.5% and 2.5% showing clear increases in calcium, magnesium and phosphorus retention compared to treatments 0% and 0.5%.

It has been shown that mineral availability is increased through the inclusion of organic acids or their salts to fish diets. For example, improved phosphorus retention has been detected in rainbow trout fed organic acids in the diet (Pandey & Satoh,

2008; Hernandez et al., 2012). Apparent digestibility of Mg, Ca and P was enhanced in rainbow trout if diets consisting of fish protein concentrate, fish meal, wheat meal and wheat starch were spiked with 10 ml / kg formic acid (Vielma & Lall, 1997). For stomach less fish (*Labeo rohita*), addition of 3% citric acid led to increased bone retention of calcium and phosphorus, but not magnesium. The authors of this study observed a significant decrease in intestinal digesta pH from 6.6 ± 0.12 to 5.7 ± 0.03 (± standard error) and attributed a concomitant higher mineral solubility in lower pH environments to better mineral availabilities (Baruah et al., 2005). However, salts of organic acids can also increase mineral digestibility as demonstrated with sodium propionate and phosphorus in *Litopennaeus vannamei* (Corrêa da Silva et al., 2013) and with citrate and phosphorus in rainbow trout (Lueckstädt, 2008). Salts of organic acids are basic in solution and therefore improvements in mineral availabilities cannot be associated with higher mineral solubility. It appears impossible at present to define the mechanisms behind improved mineral availability observed in the present trial.

On the other hand, there is the possibility that at least part of the recorded increases in calcium, phosphorus and magnesium are due to urinary stones which could accumulate and crystallize in the kidney of fish fed high-oxalate diets. About 80% of urinary stones in humans consist of insoluble calcium oxalate and calcium phosphate, while the remaining 20% consist of uric acid and calcium (10%) and struvite stones (10%), a mineral containing ammonia, magnesium and phosphate (Sargut et al., 2010). In the long term in humans, urinary stones can adversely affect kidney function and lead to renal tubular obstruction, vascular necrosis and hemorrhage involving anuria, uremia, electrolyte disturbances or rupture (Noonan & Savage, 1999). Uremia, the toxic accumulation of nitrogenous substances in the blood due to renal failure, would not be expected in carp as ammonia is mainly excreted through the gills (Brockway, 1950), however, all other symptoms should be investigated in

fish. In the present study there was no option to perform histology to screen for urinary stones in the experimental fish. Further studies involving histopathology of the carp kidney are needed to determine the exact effects of high oxalate content on the crystallization of urinary stones.

Blood plasma composition

Hypocalcaemia is one of the main symptoms of oxalate poisoning in sheep due to the binding of oxalate in the digestive tract thereby making calcium unavailable for assimilation (El Khodery, 2008; Rahman et al., 2011). No hypocalcaemia could be observed in the present trial. James & Butcher, (1972) observed increasing plasma phosphorus as well as decreasing plasma magnesium with higher oxalate content in diets for sheep and again, these tendencies could not be identified in the present trial. An apparent explanation for this could be an up-regulation of calcium and magnesium uptake by the gills to compensate for potentially lower accessibility from the feed.

Creatinine is an indicator of kidney function and if creatinine content the blood plasma of one treatment was increased relative to other treatments, this would indicate a problem with glomerular filtration (Kampmann & Hansen, 1981). There were no indications of such effects in the present trial.

Plasma cholesterol showed a tendency to increase in low-oxalate treatments. This correlates to the higher body lipid contents and underlines the increased lipid productive value of these treatments. Lower plasma cholesterol levels in fish fed diets containing high contents of plant based feedstuffs compared to fishmeal based diets have been documented (Kaushik et al., 2004). The presence of oxalate in these plant-based diets may be associated to this effect, but further studies are required in this respect.

Plasma glucose for treatments diets 0% and 0.5% also showed a tendency to be slightly elevated compared to the higher oxalate treatments, which is in line with

results by El-Khodery et al. (2008) who observed lower glucose levels in plasma of sheep fed high-oxalate diets.

Conclusion

The effects of different concentrations of dietary oxalate on common carp have not been previously investigated despite high contents of this anti-nutritional factor in popular feedstuffs. Oxalate in carp had a positive effect on growth and it is hypothesized that this may be due to antimicrobial effects exerted in the intestine. The effects on body nutrient composition were distinct, higher oxalate promoted higher mineral and lower lipid content. No anti-nutritional effects, predominantly mineral deficiencies, as described in the literature could be detected over the trial period; however, long-term studies including histopathology are required in order to conclude whether oxalate is beneficial in carp feeds.

5.1.5 References

Baruah, K., Pal, A. K., Sahu, N. P., Jain, K. K., Mukherjee, S. C., & Debnath, D. (2005). Dietary protein level, microbial phytase, citric acid and their interactions on bone mineralization of *Labeo rohita* (Hamilton) juveniles. *Aquaculture Research*, *36*(8), 803–812.

Baruah, K., Sahu, N. P., Pal, A. K., Jain, K. K., Debnath, D., & Mukherjee, S. C. (2007). Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. *Aquaculture Research*, *38*(2), 109–120.

Brockway, D. R. (1950). Metabolic products and their effects. *The Progressive Fish-Culturist*, *12*(3), 127–129.

Correa da Silva, B., Vieira, F. D. N., Mouriño, J. L. P., Ferreira, G. S., & Seiffert, W. Q. (2013). Salts of organic acids selection by multiple characteristics for marine shrimp nutrition. *Aquaculture*, *384-387*, 104–110.

El-Khodery, S., El-Boshy, M., Gaafar, K., & Elmashad, A. (2008). Hypocalcaemia in Ossimi Sheep associated with feeding on beet tops (*Beta vulgaris*). *Turkish Journal of Vetenary & Animal Sciences*, *32*(3), 199–205.

Franceschi, V. R., & Horner, H. T. (1980). Calcium oxalate crystals in plants. *The Botanical Review*, *46*(4), 361–427.

Ganguly, S., Dora, K. C., Sarkar, S., & Chowdhury, S. (2012). Supplementation of prebiotics in fish feed: a review. *Reviews in Fish Biology and Fisheries*, *23*(2), 195–199.

Gislason, G., & Ringed, E. (1996). Comparative effects of dietary Na^{+}-lactate on Arctic char, *Salvelinus alpinus* L., and Atlantic salmon, *Salmo salar* L. *Aquaculture Research*, *27*(6), 429–435.

Kampmann, J., & Hansen, J. (1981). Glomerular filtration rate and creatinine clearance. *British Journal of Clinical Pharmacology*, *12*(1), 7–14.

Jongbloed, A. W., Mroz, Z., van der Weij-Jongbloed, R., & Kemme, P. (2000). The effects of microbial phytase, organic acids and their interaction in diets for growing pigs. *Livestock Production Science*, *67*(1-2), 113–122.

Hernández, A. J., Satoh, S., & Kiron, V. (2012). Supplementation of citric acid and amino acid chelated trace elements in low-fish meal diet for rainbow trout affect growth and phosphorus utilization. *Journal of the World Aquaculture Society*, *43*(5), 688–696.

Kaushik, S. J., Covès, D., Dutto, G., & Blanc, D. (2004). Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. *Aquaculture*, *230*(1-4), 391–404.

Kumar, V., Makkar, H. P. S., & Becker, K. (2011). Detoxified *Jatropha curcas* kernel meal as a dietary protein source: growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings. *Aquaculture Nutrition*, *17*(3), 313–326.

Liasko, R., Liousia, V., Vrazeli, P., Papiggioti, O., Chortatou, R., Abatzopoulos, T. J., & Leonardos, I. D. (2010). Biological traits of rare males in the population of *Carassius gibelio* (Actinopterygii: Cyprinidae) from Lake Pamvotis (north-west Greece). *Journal of Fish Biology*, *77*(3), 570–584.

Lohaus, G., Pennewiss, K., Sattelmacher, B., Hussmann, M., & Hermann Muehling, K. (2001). Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. *Physiologia Plantarum*, *111*(4), 457–465.

Lueckstädt, C. (2008). The use of acidifiers in fish nutrition. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *3*(44), 1-8.

NRC, National Research Council (2011). *Nutrient Requirements of Fish and Shrimp*. The National Academic Press, Washington.

Noonan, S. C., & Savage, G. P. (1999). Oxalate content of foods and its effect on humans. *Asia Pacific J Clin Nutr*, *8*(1), 64–74.

Pandey, A., & Satoh, S. (2008). Effects of organic acids on growth and phosphorus utilization in rainbow trout *Oncorhynchus mykiss*. *Fisheries Science*, *74*(4), 867–874.

Rahman, M. M., Nakagawa, T., Niimi, M., Fukuyama, K., & Kawamura, O. (2011). Effects of calcium fertilization on oxalate of Napiergrass and on mineral concentrations in blood of sheep. *Asian-Australasian Journal of Animal Sciences*, *24*(12), 1706–1710.

Rahman, M. M., Abdullah, R. B., & Wan Khadijah, W. E. (2013). A review of oxalate poisoning in domestic animals: tolerance and performance aspects. *Journal of animal physiology and animal nutrition*, *97*(4), 605–614.

Ringo, E. (1991). Effects of dietary lactate and propionate on growth and digesta in Arctic charr, *Salvelinus alpinus* (L.). *Aquaculture*, *96*(3-4), 321–333.

Sargut, S. T., Sayan, P., & Kiran, B. (2010). Influence of essential and non-essential amino acids on calcium oxalate crystallization. *Crystal Research and Technology*, *45*(1), 31–38.

Storebakken, T., Shearer, K. D., & Roem, A. J. (2000). Growth, uptake and retention of nitrogen and phosphorus, and absorption of other minerals in Atlantic salmon (*Salmo salar*) fed diets with fish meal and soy-protein concentrate as the main sources of protein. *Aquaculture Nutrition*, *6*(2), 103–108.

Vielma, J., & Lall, S. P. (1997). Dietary formic acid enhances apparent digestibility of minerals in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Nutrition*, *3*(4), 265–268.

5.2 Dietary oxalate reduces potassium, calcium, manganese and zinc digestibilitiy in tilapia (*Oreochromis niloticus*)

January – April 2014

Carsten Krome[1,2], Kim Jauncey[2], Ulfert Focken[1]

[1)] Thuenen Institute of Fisheries Ecology, Ahrensburg, Germany

[2)] Institute of Aquaculture, University of Stirling, UK

Abstract

Oxalate, a plant component present in several aquaculture feed-relevant seed and leaf types, was investigated for potential anti-nutritional attributes on tilapia (*Oreochromis niloticus*). A total of four isonitrogenous and isoenergetic diets were formulated in which dietary cellulose was replaced with soluble sodium oxalate at 0%, 0.5%, 1.5% and 2.5%. Two trials were conducted, a feeding trial to evaluate impact of oxalate on growth and nutrient digestibility and a histology trial to detect potential damaging effects of oxalate on kidney tissue. For the feeding trial, diets were fed to a total of 160 fish (initial weight: 7.47 ± 0.06 g) split into twenty aquaria (eight fish per aquaria, n = 5) and fed eight times the maintenance ration for six weeks. Weight of fish quadrupled during the experimental duration with no effect of soluble oxalate on specific growth rate, feed conversion ratio, apparent dry matter or energy digestibility. Apparent digestibility of minerals showed significant negative correlations of oxalate content for potassium (p = 0.004), calcium (p = 0.000), manganese (p = 0.028) and zinc (p = 0.010). The lower digestibility of these minerals had no effect on body mineral composition of the fish, likely because of an excess of these minerals in the diet. Body lipid content was significantly influenced by oxalate (diet 0%: 60.1 ± 3.71 g / kg wet weight; diet 2.5%: 52.9 ± 4.46, p = 0.03).

For the histology trial, twenty fish (initial weight 70.6 ± 3.50 g) were kept individually in aquaria for four weeks and fed five times the maintenance ratio. No differences in kidney cross-sections between samples were observed. Blood plasma cholesterol levels in fish fed 1.5% and 2.5% oxalate showed a non-significant tendency to decrease ($p = 0.11$).

In conclusion, soluble oxalate adversely affects apparent digestibility of potassium, calcium, manganese and zinc in tilapia. High levels of oxalate influence lipid metabolism leading to lower body lipid content, presumably connected to lower blood cholesterol.

5.2.1 Introduction

In the present trial, we investigate the effects of soluble disodium oxalate on the development of juvenile tilapia (*Oreochromis niloticus*). Growth, dry matter and mineral digestibilities, nutrient and mineral retention, selected blood plasma parameters and dietary oxalate influence on kidney stone formation are discussed.

5.2.2 Material and methods

Diet Formulation

Four isonitrogeneous and isoenergetic diets were produced containing 0% (Control), 0.5%, 1.5% and 2.5% of di-sodium-oxalate (Table 5.2.1). Protein content was formulated to be 32% and lipid content 10% (50% fish oil, 50% sunflower oil). Vitamins and minerals were supplied in the form of a premix. Essential amino acids were added to meet the requirements of tilapia (NRC, 2011). Dietary ingredients were mixed with a standard kitchen blender. Di-sodium- oxalate was slowly sieved into the mix to ensure an even distribution. 40% water was added and the mix was passed through a meat mincer to form noodles, which were then crumbled and air-dried at 45° for 48 hours.

Experimental animals and setup

Two separate experiments were conducted. A growth trial to assess the effects of oxalate on growth, body composition, nutrient retention and digestibility and a histology trial on larger animals to assess the formation of kidney stones, blood parameters and bone mineralization. *Oreochromis niloticus* were obtained from Til-Aqua, Someren, Netherlands at around 1 g initial size and then reared to the experimental starting weights of the trials with commercial tilapia feed at the Thuenen Institute of Fisheries Ecology, Ahrensburg, Germany.

Growth trial

A total of 160 experimental fish (five replicates for each treatment, each replicate containing 8 fish) with size 7.47 ± 0.06 g were distributed into a total of twenty 25 L-aquarium tanks connected to a recirculation system. Water temperature was 26°C, with 1.5 L / min flow rate. Fish were acclimatized to the system for one week. The experimental duration was 8 weeks. Water quality parameters were measured on a weekly basis. Faeces were collected daily throughout the last two weeks of the experiment. During this period, tanks were cleaned every morning before the first feeding and faeces were collected in the afternoon by siphoning from the tank. When a fish died, it was removed from the tank, weighed and the feed ration was readapted to the total weight of the replicate.

Histology trial

Twenty fish (initial size: 70.6 ± 3.50 g, five single fish per treatment) were placed individually in 45 L aquariums and fed the experimental diets for four weeks with the remaining parameters being equal to the growth trial.

As a parameter for feed quantity, the metabolic maintenance ration of the fish was calculated (see 2.1.3).

Chemical analyses as well as performance calculations and and equations were conducted as outlined in Chapter 2.

Pre- and post-experimental activities

Growth trial

Before the experiment, 8 fish were sacrificed with an overdose of ethyleneglycolmonophenylether (Liasko et al., 2010) and stored in polyethylene bags at -20°C. At the end of the study, after 8 weeks, all experimental fish were sacrificed in the same manner. For further processing, fish were autoclaved and demineralised water was added. Subsequently, the fish were homogenized using an ultra turrax blending device and transferred to a pre-weighed plastic container. The homogenized material was frozen and freeze-dried. After samples were completely dry, they were weighed and again homogenized in a standard electric coffee grinder to obtain a fine powder with which subsequent analyses were conducted.

Table 5.2.1 Compositions and proximate analysis of experimental diets containing different levels of oxalate and fed to Nile tilapia in the growth and the histology trial.

Ingredient (g kg^{-1})	Control	0.5%	1.5%	2.5%
Fish meal	380.0	380.0	380.0	380.0
Wheat meal	486.4	486.4	486.4	486.4
Sunflower oil	42.0	42.0	42.0	42.0
Fish oil	12.0	12.0	12.0	12.0
Cellulose	37.50	30.00	15.00	0.00
Vitamin premix[1)]	20.0	20.0	20.0	20.0
Mineral premix[2)]	10.0	10.0	10.0	10.0
NaH_2PO_4	10.0	10.0	10.0	10.0
Na_2-Oxalat	0.00	7.50	22.50	37.50
Threonine	0.38	0.38	0.38	0.38
Histidine	0.80	0.80	0.80	0.80
Valine	0.44	0.44	0.44	0.44
Yttrium	0.50	0.50	0.50	0.50
Proximate analysis				
Crude protein	327.9	327.7	324.0	335.1
Lipid	107.6	108.3	110.3	109.2
Gross energy (kJ / g)	19.0	18.2	18.5	18.5
Protein : Energy (mg / kJ)	17.3	18.0	17.5	18.1
Minerals				
Sodium	6.2	8.5	13.3	17.6
Magnesium	2.1	2.0	1.9	1.9
Phosphorus	15.4	15.9	14.6	14.8
Potassium	6.0	5.8	5.7	5.5
Calcium	20.4	21.3	18.7	19.3
Manganese (mg / kg)	38.9	37.4	36.9	35.9
Iron (mg / kg)	233.6	221.7	200.2	189.6
Copper (mg / kg)	14.2	14.4	12.6	13.0
Zinc (mg / kg)	85.7	85.2	81.1	80.5
Cobalt (µg / kg)	375.7	416.3	452.7	390.5

Values in g / kg fresh matter unless otherwise stated.

[1)] Vitamin premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg, unless otherwise stated): Vitamin A: 500,000 I.E./kg; Vitamin D3: 50,000 I.E./kg; Vitamin E:2500; Vitamin K3 (as Menadione): 1000; Vitamin B1: 5000; Vitamin B2:5000; Vitamin B6: 5000; Vitamin B12: 5; Nicotinic acid: 25000; Pantothenic acid: 10000; Folic acid: 1000; Biotin: 250; Choline chloride: 100000; Inositol: 25000; Vitamin C: 20125.

[2)] Mineral premix for fish supplied by Altromin Spezialfutter GmbH. Composition (mg/kg mix): Calcium: 122,160; Magnesium: 14,960; Sodium: 18,180; Potassium: 210,250; Sulfur: 15,460; Chlorine: 29,720; Iron: 1220; Manganese: 1000; Zinc: 1630; Copper: 155; Iodine: 120.

Histology trial

Fish were sacrificed after the experimental period of four weeks. Directly after sacrifice fish were weighed and the caudal vein of each fish was punctured with a 1 ml syringe, blood withdrawn and centrifuged at 4500 rpm for 3 minutes at room temperature to obtain plasma. Plasma was immediately analyzed after centrifugation. Fish were cut open at the ventral side and the kidney was carefully dissected and conserved in 10% formalin solution. Tissues were processed by routine histological methods. After paraffin wax embedding, 10 µm sections were trimmed and stained with haematoxylin (1 g haematoxylin, 0.2 g sodium iodate, 50 g potassium alum, 1 g citric acid, 50 g chloral hydrate, 1000 ml dist. Water) and eosin solution (Harrison & Richards, 1979). The eosin solution was a blend of 8 parts eosin (10 g eosin, 1000 ml dist. Water) and 1 part Putt´s eosin (4 g eosin, 2 g potassium dichromate, 40 ml picric acid, 40 ml absolute alcohol, 320 ml dist. water).

Statistical analyses

Percentages were arcsine transformed before analysis. Multiple regression analysis was applied to test for correlations between different oxalate concentrations in diet and parameters tested and considered significant at $p \leq 0.05$. In the case of survival, non-parametric Kruskal-Wallis test was deployed to test for significant differences. Statistics were conducted with Statistica 8 software. Values are expressed as mean ± standard deviation.

5.2.3 Results

Growth trial

Fish consumed all diets completely at all times. Survival of fish was between 85% and 90% for diets 0%, 0.5% and 1.5%, but was lower for the high oxalate treatment (55 ± 25.9%). However, these differences were not significant due to high standard variations (Table 5.2.2). Continuous fighting behavior was observed in all tanks. Dead

fish were occasionally nibbled on by other fish in a tank before they could be removed. Tanks with mortalities higher than 50% were not included in the growth, digestibility or body composition analyses of the trial.

Table 5.2.2 Survival of Nile tilapia fed 0%, 0.5%, 1.5% and 2.5% of oxalate for 6 weeks

Control	0.5%	1.5%	2.5%
85.0 ± 13.7	90.0 ± 15.3	85.0 ± 22.4	55.0 ± 25.9

Values in %. No significant differences found between treatments (Kruskal-Wallis test, p = 0.12)

Growth of experimental fish was generally fast with specific growth rates around 3.5% / day in all treatments. There were no differences observed in final weight, specific growth rate or feed conversion ratio. Protein efficiency and productive value as well as lipid productive value was equal in all treatments (Table 5.2.3).

Oxalate content in diets had a significant negative influence on several mineral digestibilities (Table 5.2.4). Potassium (p = 0.004), calcium (p = 0.000), manganese (p = 0.028) and zinc (p = 0.010) were impaired with higher oxalate dietary oxalate concentration. A tendency for lower iron (p = 0.122) and copper (p = 0.112) digestibilities was also observed. No correlation was observed for dry matter, protein or energy digestibility.

Table 5.2.3 Growth parameters of Nile tilapia fed different dietary oxalate concentrations for 6 weeks.

Parameter	Control	0.5%	1.5%	2.5%	R^2	P
IW	7.45 ± 0.07	7.48 ± 0.02	7.49 ± 0.05	7.47 ± 0.10		
FW	31.7 ± 2.31	32.9 ± 3.25	31.6 ± 0.89	31.8 ± 0.33	0.01	0.72
SGR	3.45 ± 0.17	3.52 ± 0.10	3.42 ± 0.06	3.45 ± 0.06	0.01	0.71
FCR	1.16 ± 0.10	1.10 ± 0.11	1.09 ± 0.12	1.11 ± 0.17	0.02	0.61
PER	2.63 ± 0.21	2.78 ± 0.27	2.85 ± 0.31	2.72 ± 0.42	0.01	0.68
PPV	35.9 ± 3.65	35.0 ± 3.03	35.2 ± 2.97	32.0 ± 3.11	0.02	0.59
LPV	48.8 ± 3.90	56.1 ± 10.8	55.6 ± 7.05	41.9 ± 2.46	0.11	0.23

N = 5. IW: Initial weight (g); FW: Final weight (g); SGR: Specific growth rate (% / day); FCR: Feed conversion ratio; PER: Protein efficiency ratio; PPV: Protein productive value (%); LPV: Lipid productive value (%). No correlation between treatments observed.

Table 5.2.4 Apparent dry matter, protein and mineral digestibilities of Nile tilapia fed different dietary oxalate levels for 6 weeks.

Treatment	Control	0.5%	1.5%	2.5%	R^2	P
Dry matter	73.2 ± 2.08	69.2 ± 3.89	70.4 ± 4.34	69.1 ± 3.51	0.10	0.186
Protein	87.2 ± 1.98	87.0 ± 2.88	84.5 ± 5.21	85.8 ± 2.49	0.06	0.310
Energy	77.3 ± 1.91	75.3 ± 2.69	76.4 ± 3.26	78.1 ± 3.64	0.03	0.555
Magnesium	73.4 ± 5.00	64.5 ± 10.5	66.6 ± 8.10	70.5 ± 6.10	0.00	0.883
Potassium	97.7 ± 0.16	97.0 ± 0.4	96.3 ± 0.94	96.4 ± 0.60	0.38	0.004
Phosphorus	42.9 ± 7.8	40.6 ± 10.9	40.1 ± 10.2	39.0 ± 11.6	0.02	0.565
Calcium	9.3 ± 15.9	3.2 ± 19.7	-15.6 ± 18.4	-26.2 ± 21.3	0.42	0.000
Manganese	-9.0 ± 18.2	-25.8 ± 25.1	-54.3 ± 38.4	-55.9 ± 51.4	0.27	0.028
Iron	10.1 ± 18.9	3.8 ± 25.7	0.2 ± 14.8	-9.9 ± 15.0	0.13	0.122
Copper	50.4 ± 11.1	43.0 ± 17.6	36.8 ± 9.3	37.0 ± 13.5	0.14	0.112
Zinc	7.7 ± 3.50	-1.3 ± 17.0	-27.9 ± 28.4	-21.9 ± 19.1	0.31	0.010
Cobalt	72.2 ± 2.80	70.3 ± 3.90	74.1 ± 3.90	72.2 ± 3.00	0.00	0.916

Values in %, n = 5, Values with $p \leq 0.05$ show a significant correlation.

There was a negative correlation of body lipid content ($p = 0.03$) with treatment 2.5% showing the lowest (5.29 ± 0.45% wet weight) compared to other treatments (Table 5.2.5). There were no significant differences in body moisture, protein or ash. Lower digestibility of some minerals also had no influence on body mineral concentrations. On the contrary, manganese actually showed higher body contents with higher oxalate concentrations in the diet ($p = 0.02$, Table 5.2.5)

Table 5.2.5 Body nutrient and mineral composition of Nile tilapia fed different dietary oxalate levels for 6 weeks.

Treatment	Control	0.5%	1.5%	2.5%	R^2	P
Moisture	757.6 ± 11.4	759.2 ± 3.65	756.8 ± 5.09	765.0 ± 8.20	0.08	0.21
Crude Protein	129.7 ± 4.92	129.3 ± 3.06	129.3 ± 1.59	131.3 ± 6.25	0.00	0.89
Crude Lipid	60.1 ± 3.71	61.5 ± 3.60	61.4 ± 6.20	52.9 ± 4.46	0.24	0.03
Ash	36.5 ± 2.56	35.7 ± 1.32	36.9 ± 1.33	37.0 ± 1.66	0.04	0.40
Minerals						
Sodium	1.17 ± 0.08	1.16 ± 0.06	1.05 ± 0.10	1.18 ± 0.05	0.01	0.72
Magnesium	0.30 ± 0.02	0.31 ± 0.02	0.30 ± 0.03	0.31 ± 0.01	0.00	0.97
Phosphorus	5.94 ± 0.43	6.19 ± 0.43	6.06 ± 0.68	6.33 ± 0.37	0.04	0.39
Potassium	2.61 ± 0.19	2.56 ± 0.10	2.39 ± 0.21	2.64 ± 0.19	0.01	0.78
Calcium	9.37 ± 0.77	9.78 ± 0.84	9.60 ± 1.20	10.1 ± 0.59	0.05	0.35
Manganese (mg / kg)	2.51 ± 0.20	2.73 ± 0.28	3.07 ± 0.48	3.22 ± 0.77	0.28	0.02
Iron (mg / kg)	20.4 ± 2.09	21.5 ± 3.52	22.9 ± 2.82	23.1 ± 4.07	0.11	0.18
Copper (mg / kg)	10.3 ± 2.57	11.8 ± 3.90	12.1 ± 1.88	12.0 ± 1.51	0.04	0.42
Zinc (mg / kg)	15.1 ± 1.28	16.0 ± 1.57	16.8 ± 0.96	15.8 ± 1.00	0.04	0.46
Cobalt (µg / kg)	13.6 ± 1.39	13.8 ± 3.79	14.2 ± 3.63	15.5 ± 2.72	0.04	0.44

Values based on fresh matter and given in g / kg, unless otherwise stated, n = 5, $p \leq 0.05$.

Histology trial

Fish consumed all diets completely at all times. Survival was 100% for all treatments. Fish on average doubled in body weight in the four experimental weeks.

Kidney cross-sections showed calcareous structures in all treatments at an equal amount (Figure 5.2.1). Where these structures were observed they lead to dilation of the tubule. The lesion caused were considered not numerous and severe enough to affect health.

There was a tendency towards lower cholesterol in blood plasma of fish fed 1.5% and 2.5% of oxalate, however, this was not significant (p = 0.11). No correlation was found in plasma calcium or glucose (Table 5.2.6)

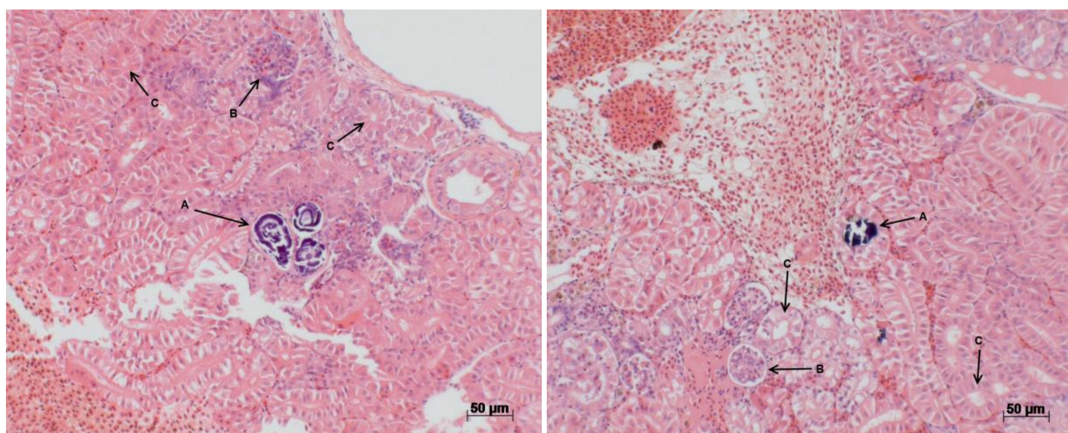


Figure 5.2.1 Kidney cross-sections of Nile tilapia fed 0% oxalate (left) and 2.5% oxalate (right) for 4 weeks.

A = Calcareous body formation
B = Glomerulus
C = Convoluted tubule

Table 5.2.6 Selected blood plasma values of Nile tilapia of the histology trial fed experimental diets for 4 weeks.

Treatment	Control			0.5%			1.5%			2.5%			R^2	P
Calcium	13.8	±	1.08	12.7	±	1.58	12.8	±	1.27	12.4	±	1.68	0.12	0.23
Glucose	69.8	±	21.0	61.0	±	18.9	62.3	±	5.13	58.8	±	5.74	0.06	0.38
Cholesterol	121.5	±	12.7	124.0	±	7.81	96.7	±	17.0	108.0	±	18.9	0.20	0.11

Values in mg / dl, n = 5. No correlation between treatments observed.

5.2.4 Discussion

The present work was originally designed to investigate potential adverse effects of soluble oxalate on growth of tilapia. It was motivated by an earlier study describing 2.44% of soluble oxalate in detoxified *Jatropha curcas* kernel meal, which when added to diets for tilapia or carp led to slower growth rates (Krome et al., 2014). The present results showed no adverse effects of high oxalate inclusions on specific growth rate or feed conversion ratio. Specific growth rates between 3.42 ± 0.06 % / day (1.5%) to 3.52 ± 0.10% / day (0.5%) were very high compared to equivalent diets in comparable experimental set-ups for tilapia (Kumar et al., 2012; Krome et al., 2014). Survival of fish was not significantly different between treatments, however, a tendency towards lower survival of fish fed 2.5% oxalate could be observed (p

= 0.12). This could have been due to oxalate poisoning, a condition that can either be acute or chronic and has been studied for a variety of livestock (Rahman et al., 2013). In the acute form, blood plasma calcium levels abruptly decrease, disrupting energy metabolism in the cell and leading to muscle tremors, weakness, consequential collapse and eventual death (Rahman et al., 2013). Chronic oxalate poisoning results through steady precipitation of calcium oxalate in the blood stream and filtration of calcium oxalate crystals through the kidney. This causes damage to the kidney tubules, which eventually leads to kidney failure (Rahman et al., 2013). Data on acute toxicity levels of dietary oxalate have been reported for ruminants to be around 3% (Sidhu et al., 2001), but may also be lower (Marais et al., 2001). The authors of the present trial could not find data on fish on that matter. Whether the reason behind the presumably higher mortalities in the high oxalate diet of the present trial was due to oxalate poisoning cannot be confirmed with the present data. Blood plasma calcium showed no significant correlation to diet oxalate. Kidney cross-section samples showed no differences in the abundance and severety of stone formations between treatments. Stone formations as seen in this trial were similar to nephrocalcinosis observed in trout by Harrison & Richards (1979) or tilapia by Chen et al. (2001). Cross-sections of kidney from these works showed larger-sized and more frequent lesions compared to the present trial. Bunce et al. (1980) consistently induced renal calcification in rats when feeding a diet with a high calcium / magnesium ratio and attributed the effects to the stabilizing function of the magnesium ion in the glomerular filtrate. Diets of the present trial contained around 0.2 g / kg magnesium, which is equal to the required amount of juvenile tilapia in freshwater (Lin et al., 2013). The optimum dietary calcium concentration of 4 g / kg (Shiau & Tseng, 2007), however, was clearly exceeded (around 20 g / kg). Therefore calcified structures observed in the kidneys are likely to derive from an inade-

quately high calcium / magnesium ratio in diets of the present trial rather than differences in oxalate content.

The present dataset revealed a significant adverse influence of dietary oxalate concentration on potassium, calcium, manganese and zinc digestibility. Mineral balance of non-ruminant livestock has been known to be disturbed through dietary oxalate (Libert & Franceschi, 1987). In general, ruminants seem to utilize minerals in oxalate-rich sources better than non-ruminants due to oxalate-degrading bacteria in the rumen (Rahman et al., 2013). In a trial conducted on rats, four different diets were designed with different levels of Amaranth (*Amaranthus gangeticus*), a plant with soluble oxalate contents of 3.8% to 5.0% (Larsen et al., 2003). Results showed lower calcium availability with higher inclusion levels of the plant and consequential reduced lower femur bone calcium and total femur bone mass in relation to body weight. In their study, lower calcium availability had no influence on growth or blood plasma calcium, which is similar to the present study (Larsen et al., 2003). A study on cattle revealed 20% lower availability of calcium when oxalate-rich grass was consumed compared to oxalate-poor grass, while no differences for magnesium or phosphorus availability was observed (Blaney et al., 1982). Lower digestibility of the mentioned minerals did not seem to have an effect on fish development in the present trial.

It is generally understood that the bioavailability of oxalate or oxalate-bound minerals is low if precipitated. The degree of precipitation in the digestive tract to a large extent depends on the amount of divalent cations present (Liebman & Al-Wahsh, 2011). Calcium oxalate is practically insoluble in water (6 mg / L at 18°C, Libert & Franceschi, 1987) and this is thought to be only marginally influenced by varying pH values prevalent in the digestive tract (Liebman & Al-Wahsh, 2011). The concentration of oxalate in solution in the digestive tract is therefore strongly dependent on the molar oxalate calcium ratio. In the present trial, all experimental feeds contained

calcium in excess through a mineral supplement as well as fish meal (around 20 g / kg calcium in feed). The molar oxalate calcium ratio in diet 2.5% was therefore around 0.56. It is likely that at a ratio lower than one, most oxalate is bound to calcium, but only slightly more than half the dietary calcium is chelated with the remainder available for absorption. The optimum dietary calcium concentration for juvenile tilapia reared in fresh water was shown to be around 4 g / kg (Shiau & Tseng, 2007), which would still leave more than double the optimum concentration available to the fish even provided that 100% of oxalate is bound to calcium and not available for absorption. This would also explain why no impact of lower calcium digestibility on whole body calcium content could be observed (Table 5.2.5). Though tilapia may also regulate calcium homeostasis through specialized cells (ionocytes) in the gills (Flik & Verbost, 1993), which contributes to calcium supply for the fish, Shiau & Tseng (2007) showed that dietary calcium supply in low-calcium fresh water is essential in order to achieve growth rates comparable to what has been observed in the present trial. A more typical commercial diet for tilapia with fishmeal inclusion around 5% (Tacon & Metian, 2008) and the remainder coming from plant-based protein sources, such as *Jatropha curcas* kernel meal would imply lower total dietary calcium contents as calcium content of plant feedstuffs are typically lower than fishmeal (calcium content of fishmeal: 22 – 79 g / kg (NRC, 2011); in comparison: calcium content of *Jatropha curcas* kernel meal: 8 g / kg, measured by ICP-MS, Institute of Aquaculture, Stirling, UK). The same counts for zinc (NRC, 2011). It may be hypothesized that lower mineral digestibilities through high soluble oxalate concentrations in largely plant-based diets will have to be taken into account when computing the necessary mineral supplementation, especially with regards to calcium and zinc (Do Carmo e Sá et al., 2004; Shiau & Tseng, 2007).

Body composition of fish in the present trial shows no reductions in mineral composition. As mentioned before, this is likely due to more than sufficient supply even of

those minerals that show adverse effects of digestibility through oxalate. Interestingly, body manganese concentration is significantly higher in fish fed diet 2.5%, despite lower manganese digestibility. Non-dietary manganese uptake is possible (Watanabe et al., 1997). In what way oxalate influences manganese incorporation is not known. Manganese is an important co-factor for enzymes that form metal-enzyme complexes as well as an integral component of metalloenzymes (Watanabe et al., 1997).

The present trial demonstrates an impact of dietary oxalate on fish lipid metabolism. There was an inverse relationship between oxalate and body lipid content of fish. Body lipid content seems to be significantly reduced only in diet 2.5%. Lower blood plasma cholesterol values observed in the higher oxalate treatments during the histology trial support these findings (Table 5.2.6). The results are coherent with a similar experiment on carp, in which fish fed 1.5% and 2.5% soluble oxalate showed significantly lower body lipid than lower concentrations (Krome et al., unpublished). Oxalate is the salt of oxalic acid, an organic acid, and these have been known to interfere with the intermediary metabolism of animals (Partanen & Mroz, 1999). Baruah et al. (2007) found citric acid to reduce body lipid content by 16.5% when added to diets for *Labeo rohita* juveniles at a 3% inclusion. What the exact mechanisms are behind the reduction of body lipid through oxalate inclusion cannot be answered at this point.

Conclusion

The present trial shows that dietary soluble oxalate has adverse effects on apparent digestibility of potassium, calcium, manganese and zinc. In the present set-up, this did not have any significant effects on tilapia growth or body mineral composition, probably due to excess minerals provided with the diet. A reduction of body lipid and presumably plasma cholesterol is documented through oxalate. It remains unclear whether oxalate also shows toxic effects and whether mineral deficiency signs

would appear in a more plant-based diet with lower mineral content. Further research is required in this respect. To our knowledge, this is the first report showing the influence of soluble oxalate on mineral digestion in fish.

5.2.5 References

Akinleye, A. O., Kumar, V., Makkar, H. P. S., Angulo-Escalante, M. A., & Becker, K. (2011). *Jatropha platyphylla* kernel meal as feed ingredient for Nile tilapia (*Oreochromis niloticus* L.): growth, nutrient utilization and blood parameters. *Journal of Animal Physiology and Animal Nutrition*, *96*(1), 1–11.

Baruah, K., Sahu, N. P., Pal, A. K., Jain, K. K., Debnath, D., & Mukherjee, S. C. (2007). Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. *Aquaculture Research*, *38*(2), 109–120.

Blaney, B. J., Gartner, R. J. W., & Head, T. a. (1982). The effects of oxalate in tropical grasses on calcium, phosphorus and magnesium availability to cattle. *The Journal of Agricultural Science*, *99*(3), 533–539.

Bunce, G. E., Saacke, R. G., & Mullins, J. (1980). The morphology and pathogenesis of magnesium nephrocalcinosis. *Experimental and Molecular Pathology*, *33*, 203–210.

Chen, C., Wooster, G. A., Getchell, R. G., Bowser, P. R., & Timmons, B. (2001). Nephrocalcinosis in Nile Tilapia from a recirculation aquaculture system : A case report. *Journal of Aquatic Animal Health*, *13*, 368–372.

Do Carmo e Sá, M. V., Pezzato, L. E., Ferreira Lima, M. M. B., & de Magalhães Padilha, P. (2004). Optimum zinc supplementation level in Nile tilapia *Oreochromis niloticus* juveniles diets. *Aquaculture*, *238*(1-4), 385–401.

Flik, G., & Verbost, P. M. (1993). Calcium transport in fish gills and intestine. *Journal of Experimental Biology*, *184*, 17–29.

Harrison, J. G., & Richards, R. H. (1979). The pathology and histopathology of nephrocalcinosis in rainbow trout *Salmo gairdneri* Richardson in fresh water. *Journal of Fish Diseases*, *2*, 1–12.

Kim, K. H., Lohakare, J. D., Sen, S., Ingale, S. L., Singh, N. K., Park, C. K., … Chae, B. J. (2011). Comparison of growth performance, nutrient digestibility, blood metabolites and carcass traits in Korean native and crossbred weaning piglets. *Animal Nutrition and Feed Technology*, *11*(2), 159–167.

Krome, C., Jauncey, K., Fedderke, S., & Focken, U. (2014). Effect of replacing different levels of dietary fishmeal with *Jatropha curcas* kernel meal on the development of Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758). *Journal of Applied Ichthyology*, *30*(3), 507–512.

Kumar, V., Akinleye, A. O., Makkar, H. P. S., Angulo-Escalante, M. A., & Becker, K. (2012). Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing *Jatropha platyphylla* kernel meal as a protein source. *Journal of Animal Physiology and Animal Nutrition*, *96*(1), 37–46.

Larsen, T., Thilsted, S. H., Biswas, S. K., & Tetens, I. (2003). The leafy vegetable amaranth (*Amaranthus gangeticus*) is a potent inhibitor of calcium availability and retention in rice-based diets. *British Journal of Nutrition*, *90*, 521–527.

Liasko, R., Liousia, V., Vrazeli, P., Papiggioti, O., Chortatou, R., Abatzopoulos, T. J., & Leonardos, I. D. (2010). Biological traits of rare males in the population of *Carassius gibelio* (Actinopterygii: Cyprinidae) from Lake Pamvotis (north-west Greece). *Journal of Fish Biology*, *77*(3), 570–584.

Libert, B., & Franceschi, V. R. (1987). Oxalate in crop plants. *Journal of Agricultural and Food Chemistry*, *35*(6), 926–938.

Liebman, M., & Al-Wahsh, I. A. (2011). Probiotics and other key determinants of dietary oxalate aborption. *Advances in Nutrition*, *2*, 254–260.

Lin, Y.-H., Ku, C.-Y., & Shiau, S.-Y. (2013). Estimation of dietary magnesium requirements of juvenile tilapia, *Oreochromis niloticus* × *Oreochromis aureus*, reared in freshwater and seawater. *Aquaculture*, *380-383*, 47–51.

NRC, National Research Council (2011). *Nutrient Requirements of Fish and Shrimp*. The National Academic Press, Washington.

Partanen, K. H., & Mroz, Z. (1999). Organic acids for performance enhancement in pig diets. *Nutrition Research Reviews*, *12*(1), 117–145.

Rahman, M. M., Abdullah, R. B., & Wan Khadijah, W. E. (2013). A review of oxalate poisoning in domestic animals: tolerance and performance aspects. *Journal of Animal Physiology and Animal Nutrition*, *97*(4), 605–614.

Shiau, S.-Y., & Tseng, H.-C. (2007). Dietary calcium requirements of juvenile tilapia, *Oreochromis niloticus* x *O. aureus*, reared in fresh water. *Aquaculture Nutrition*, *13*(4), 298–303.

Tacon, A. G. J., & Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture*, *285*(1-4), 146–158.

Watanabe, T., Kiron, V., & Satoh, S. (1997). Trace minerals in fish nutrition. *Aquaculture*, *151*(1-4), 185–207.

6 General Discussion

Up to now, the press cake accumulating after oil extraction of *Jatropha curcas* seeds (JKM) has been disposed as fertilizer or pelleted solid fuel. The existence of toxic phorbol esters has limited its application as a feedstuff despite a high protein content and balanced amino acid composition. The recently patented detoxification process (Makkar & Becker, 2011) has paved the way for the use of JKM as an ingredient in animal diets. The higher value of JKM as a feedstuff compared to former uses would be important step in improving the economic viability of *Jatropha curcas* plantations (Makkar & Becker, 2009).

Originally, a field trial was planned with a partnering institute in Egypt a well as an industrial feed producer to assess the practical suitability of JKM for carp and tilapia. This would have had several advantages:

1. In a natural environment, JKM as a feed ingredient could yield different results compared to aquarium or tank based trials as conducted in this thesis. Natural food sources like phytoplankton and zooplankton significantly influence growth rates and yield in carp and tilapia pond culture (Sparatu et al., 1983) and this is influenced through type and quantity of feed (Bechara et al., 2005).

2. An extruded diet, undergone pressure and heat treatment during the extrusion process, may have different nutritional and physical properties than a diet produced in the laboratory. Especially starch digestibility may be improved (Pfeffer et al. 1991).

3. Longer-term trials lasting for the majority of the grow-out cycle could yield significantly different results than 8-week trials as conducted in most experiments of this

thesis. This is because of long-term positive or negative effects JKM may have on growth rates, FCR or health condition of fish (Figure 3.2.2, Lee et al., 2006).

Unfortunately, the required amounts of JKM for a large-scale field trial could not be supplied. Because of that and the development of the political situation in Egypt during the time of this thesis, the field trial had to be cancelled.

In the following key findings of previous research are summarized and related to the findings of the present work.

6.1 Growth of carp and tilapia fed diets containing different levels of JKM

Chapter 3 of the present work deals with the replacement of fishmeal protein with JKM. In Trial 3.1, it was found for carp that 50% fishmeal protein replaced by JKM was as efficient as the fishmeal control diet, if incubated with citric acid and phytase beforehand, while the 100% fishmeal replacement remained clearly lower with and without incubation. The experiment showed that phytase incubation of JKM for carp only improves growth performance at a 50% replacement level.

Measurement of phytate content of the feeds showed 0.69% and 1.33%, respectively left-over phytate to be present in the two pre-incubated diets. However, it was later observed that the method used to measure phytate in this trial was invalid and that the same concentration and type of phytase completely hydrolyses phytate in incubated JKM (Chapter 4.3, Table 4.3.3). We can therefore assume that both feeds contained no phytate. This would indicate other anti-nutritional factors playing a role in JKM that did not have an impact at a replacement level of 50%, but led to lower growth rates at 100%.

In a trial by Kumar et al. (2011a) carp were fed diets containing 50% and 75% of dietary protein respectively supplied by (non-incubated) JKM. Dietary protein content was 38% and fish (initial weight: 3.2 ± 0.07 g) were fed five times their mainte-

nance requirement, similar to the present work. Results revealed 50% replacement to have equal growth rates compared to the control, while 75% inclusion level was significantly lower. In absolute values, Trial 3.1 showed higher FCRs of the incubated 50% treatment compared to the work conducted by Kumar et al. (1.20 ± 0.12 and 1.01 ± 0.02, respectively). Trial 3.1 had a higher starting weight of the fish, which in part could explain higher FCRs. The relative results of the present trial compared the trial by Kumar et al. can be considered similar.

Trial 3.3 showed equal growth of carp to a control diet when fed a diet containing mixed plant-ingredients and only 25% of protein supplied by fishmeal. This FM based fraction could be completely replaced by JKM without decreases in growth rates or increases in FCR. However, FCR here was quite high, 1.75 ± 0.09, in control samples. Deng et al. (2011) observed FCRs of 1.67 ± 0.01 in common carp fed a diet with the same protein content containing about the same amount of fishmeal and the remainder coming from soybean and wheat meal. Differences in absolute FCRs between the present trials and previous work can arise from (aside feed composition) feeding frequency, feeding technique (hand-fed opposed to automatic feeders) or feed amount (satiation or percentage body weight). When comparing Trial 3.1 and 3.3, it can be argued that anti-nutritional factors of JKM were not present in high enough concentrations in Trial 3.3 to negatively influence growth of carp due to the lower inclusion level in this trial.

For tilapia, Trials 3.2 and 3.4 showed growth to be lower for all replacement levels of dietary fishmeal. Trial 3.4 revealed an inverse linear relationship between fishmeal replacement and specific growth rate. Unlike for carp, anti-nutritional factors seemed to have an inversely proportional effect on tilapia and affected growth already at low inclusion levels.

Not much previous work has been conducted by other authors on detoxified JKM use in tilapia diets. Growth performance parameters did not change significantly

compared to a fishmeal based control diet when tilapia were fed up to 62.5% fishmeal replacement of *Jatropha platyphylla* kernel meal, a non-toxic species endemic in Central America (Akinleye et al., 2011; Kumar et al., 2012). In absolute numbers, however, the 100% replacement level of Trial 3.2 showed better FCRs than 62.5% replacement level of the cited trials (1.28 ± 0.08 versus 1.70 ± 0.06 and 1.70 ± 0.05, respectively).

The present work shows that the addition of phytase if additional phosphate was supplied had no impact on growth (Trial 4.1). Phosphate was supplemented through the mineral premix in Trial 3.2 and Trial 3.4 and abundant in sufficient, available amounts. However, we could also show that phytate content of JKM-based diets was only reduced by 56% *in vivo* (Chapter 4.3); implying residual phytate may have been in the digestive tract reducing amino acid digestibility and potentially growth (Spinelli et al., 1983, Kumar et al., 2011c). Therefore, phytate cannot be ruled out as a potential anti-nutritional factor in these trials.

On a general note, fishmeal may also possess growth-promoting attributes that are reduced with increasing supplementation of plant-based protein. These include taurin (Gaylord et al., 2007) and possibly steroids (Hardy, 2010) and could be part of the reason why slower growth was observed in the some of the present trials.

6.2 Body composition of carp and tilapia with different levels of JKM in the diet

6.2.1 Protein

A comparison of fish body composition across all growth trials (Chapter 3) can be seen in Table 6.2.1. Fish protein content showed to be similar for both species throughout all inclusion levels of JKM. It is interesting to note that significant differences in body protein content in Trials 3.1 are not observed any longer when cor-

rected for moisture content. The reason for that is the depency of moisture on body lipid content: Fat tissue incorporates less water than muscle tissue and fattier fish will therefore have higher dry matter content leading to misleading results when not correcting for moisture content in the analysis. The same problem can be found in the work by El-Saidy & Saad (2011).

Table 6.2.1 Body composition of carp and tilapia in accordance to diet type and fishmeal replacement level of JKM – comparison across trials.

Diet Type	FM protein replacement level	Species	Moisture	CP	CL	CA
Ideal Trial 3.1 Trial 3.2	0%	Carp	76.0 ± 0.92	15.1 ± 0.30	6.13 ± 0.58	2.26 ± 0.16
		Tilapia	75.9 ± 0.77	14.9 ± 0.70	5.31 ± 0.39	3.83 ± 0.16
	50%	Carp	78.2 ± 0.38	14.8 ± 0.44	4.04 ± 0.39	2.84 ± 0.56
		Tilapia	76.0 ± 0.44	15.9 ± 0.54	4.61 ± 0.25	4.01 ± 0.09
	75%	Carp	-	-	-	-
		Tilapia	75.7 ± 0.61	15.2 ± 0.39	4.73 ± 0.53	4.07 ± 0.03
	100%	Carp	76.9 ± 0.31	15.8 ± 0.75	3.65 ± 0.81	3.97 ± 0.36
		Tilapia	75.8 ± 0.63	15.5 ± 0.47	3.97 ± 0.34	4.33 ± 0.11
Practical Trial 3.3 Trial 3.4	0%	Carp	78.1 ± 0.16	14.1 ± 0.43	4.69 ± 0.27	2.43 ± 0.10
		Tilapia	76.3 ± 0.32	15.1 ± 0.56	4.46 ± 0.47	3.67 ± 0.14
	30%	Carp	78.1 ± 1.40	13.7 ± 0.73	5.09 ± 0.71	2.32 ± 0.14
		Tilapia	76.6 ± 0.92	14.5 ± 0.74	4.85 ± 0.34	3.61 ± 0.11
	70%	Carp	77.4 ± 0.11	14.2 ± 0.10	5.52 ± 0.24	2.39 ± 0.06
		Tilapia	75.5 ± 1.24	14.9 ± 0.62	6.00 ± 0.84	2.98 ± 0.10
	100%	Carp	77.5 ± 1.02	13.9 ± 0.26	5.40 ± 0.92	2.31 ± 0.04
		Tilapia	75.9 ± 1.30	15.1 ± 0.30	5.87 ± 0.95	2.91 ± 0.07

Values in % fresh weight.
Diet type "Ideal" refers to a diet where fishmeal provided the only protein source (Trials 3.1 and 3.2), diet type "Practical" refers to a diet where several protein sources were provided (Trials 3.3 and 3.4).

6.2.2 Lipid

For the trials replacing fishmeal of a standard fishmeal diet with different concentrations of JKM (Trials 3.1 and 3.2), a tendency of decreasing body lipid with increasing inclusion level could be observed. For carp, values went from 6.13 ± 0.58% of control fish to 4.04 ± 0.39% and 3.65 ± 0.81% for 50% and 100% inclusion level, re-

spectively. Tilapia showed a similar trend (Control: 5.31 ± 0.39%; 50%: 4.61 ± 0.25%; 100%: 3.97 ± 0.34%). These results are contradictory to other works, which observed increasing body lipid with higher inclusion levels of JKM for carp (Kumar et al., 2011a, 2011b) and no differences for tilapia (Akinleye et al., 2011; Kumar et al., 2012). In line with the present observations stands a trial on gibel carp (*Carassius auratus gibelio*) using various, single plant feedstuffs as a 100% replacement for fishmeal protein: Only rapeseed cake showed similar body lipid concentration to the fishmeal control diet, while cottonseed cake, peanut cake, potato protein concentrate and soybean cake all showed lower lipid contents (Xie et al., 2001). A body lipid and cholesterol reducing impact of plant-based components, such as phytoestrogen or saponins has been known to exist (Ali et al., 2004; Chen et al., 2011; Couto et al., 2014). Untreated JKM has a saponin content of 2.6% (Table 1.3.4), which is higher than soybean meal (0.67%, Ireland et al., 1987) and this is not reduced through heat treatment (Ireland et al., 1987). Further, a high concentration of soluble oxalate was observed in JKM (Table 4.3.2). Oxalate has shown to have a body lipid reducing effect (Chapter 5). Both these components may have reduced body lipid content in these trials.

Fish fed diets, which contained a mixture of different plant feedstuffs and only 25% of protein through fishmeal (Trials 3.3 and 3.4) showed higher lipid values with increasing replacement level of JKM. To our knowledge, there have not been any previous experiments with JKM inclusion in mixed plant feedstuff diets. El Saidy & Gaber (2003) found no differences in body lipid between a fishmeal based control diet and gradually increasing amounts of a plant protein mix in tilapia. Mazurkiewicz (2009) observed higher body lipid content in juvenile common carp with higher inclusion levels of a legume - rapeseed mix. Similar results were also reported by Hasan et al. (1997), who investigated a variety of plant-based feedstuffs in carp and

also showed extensive lipid deposition in the liver and attributed these effects to substances in the feed disturbing lipid metabolism.

Along with the present data, it can be assumed that JKM contains substances (e.g. phytoestrogens, saponin, oxalate) reducing body lipid if they reach a certain concentration in the diet. If this concentration is not reached, body lipid increases, due to similar reasons to what has been observed by other researchers.

6.2.3 Ash

The opposite could be observed for body ash content of experimental fish. While those fish fed JKM as a replacement for only fishmeal as a protein source (Trials 3.1 and 3.2) showed higher body ash content with increasing JKM inclusion, fish in Trials 3.3 and 3.4 showed equal and lower ash content with increasing inclusion levels. Kumar et al. (2011a, 2011b, 2012) found no differences in body ash content for carp or tilapia for several inclusion levels from 50% to 75%. El Saidy & Gaber (2003) found significantly decreased body ash content for diets containing 75% or 100% mixed plant feedstuffs compared to lower inclusion levels. These results are congruent with Trials 3.3 and 3.4. The availability of minerals may depend on anti-nutritional factors present in plant-based feedstuffs such as phytate (Chapter 6.3) and therefore higher inclusion levels of these may lead to lower availabilities.

6.3 Influence of phytate and application of phytase in JKM-based diets

Trial 4.3 demonstrates the invalidity of the photometric methods previously used for phytate analysis of JKM. It further showed the possibility of completely reducing phytate through preincubation with phytase. In contrast to that, *in vivo* application of

phytase in JKM based diets for tilapia, revealed only a 50% decrease of phytate concentration in faeces compared to fish fed a control diet.

Chapter 3.1 investigated the impact of phytase on preincubated, JKM-based diets for carp. Results suggest a significant improvement of growth parameters for phytase-incubated diets at a fishmeal replacement level of 50%, but no positive effects of phytase- incubation at replacement levels of 100%. Improved growth parameters for carp fed phytase-incubated diets based on different plant feedstuffs were also reported by Nwanna et al. (2007).

Trials 4.1 and 4.2 dealt with the effects of phytate and phytase on tilapia fed JKM-based diets. In 4.1, it could be shown that a single phytase at 2000 U / kg has no impact on protein utilization if additional phosphorus is provided. Trial 4.2 showed that phytase increases growth when no additional phosphorus is added, but only to a limited extent and not significantly. This is probably due to the already high content of available phosphorus in JKM (see Chapter 4.3), so the liberation of additional phosphorus only produces a small effect. However, growth in this trial was very slow and the very high body lipid content of experimental fish suggests a nutritional imbalance, presumably due to a suboptimal amino acid composition. It must be said that the effects of additionally available phosphorus in Trial 4.2 might have been more pronounced at higher growth rates. Trial 4.2 further showed a very significant improvement of phosphorus retention and reduction of phosphorus load of the effluent water when phytase was applied to non-phosphorus supplemented diet. Based on the given results, several recommendations may be given:

1. Phytate analysis of JKM should not be conducted by photometric methods using the Wade-reagent. Instead methods HPIC or indirect methods should be applied (Chapter 4.3).

2. Concerning JKM or JKM based diets, respectively, phytate or inositol phosphate (IP) reduction through phytase in stomach-simulating *in vitro* conditions cannot be

put on a level with reduction *in vivo*. Phytate reduction is lower *in vivo* in tilapia. Tests of phytase efficiency can be conducted *in vivo* by measuring IP concentrations of the faeces (Chapter 4.3). If complete IP reduction *in vivo* was possible, phosphorus utilization could be further optimized.

3. Phytase-incubation with citric acid of JKM leads to improved growth rates in carp at a 50% fishmeal replacement level compared to non-incubated JKM at the same inclusion level (Trial 3.1). For carp, JKM should be pre-incubated with phytase at the ideal pH required by the enzyme. An application of a neutral phytase could be an alternative (Liu et al., 2011).

4. JKM-based diets for tilapia do not need additional phosphorus supplementation if phytase is added to the diet (Chapter 4.2). Adding a single phytase to phosphorus supplemented diets has no extra effect on fish development. There is evidence that the application of two differences types of phytase to JKM-based diets has an impact on protein utilization, but further research is required to confirm this (Chapter 4.1). In light of results of Trial 4.3 showing that phytase applied in a diet for tilapia only decreases phytate content by around 56% in the faeces, it must be said that results of Trial 4.1 and Trial 4.2 could have provided different results if diets had been pre-incubated before. A recent work by Morales et al. (2013) observed a decrease of 57% in phytate-bound phosphorus in the stomach of sea bream (*Sparus aurata*) and no further reduction in proximate and distal intestine. This is congruent with the present results and also implies that optimal availability of phytate-phosphorus and potentially bound amino acids could necessitate a pre-treatment of feedstuffs or more effective enzymes.

6.4 Influence of dietary oxalate on growth and body composition of common carp and tilapia.

The anion profile of the acid extract of JKM measured with ion chromatography about mid-way through the project (Table 4.3.2) revealed high soluble oxalate contents in the meal of 2.44% compared to soybean meal (0.20%). Trial 5.1 and 5.2 were designed to assess the nutritional or anti-nutritional effects of dietary oxalate on carp and tilapia development respectively. Diets with protein coming from fish meal were formulated with concentrations of 0%, 0.5%, 1.5% and 2.5% of soluble oxalate. The results of both trials showed very different effects of oxalate on carp and tilapia. While expected effects were seen for tilapia (lower calcium, zinc, manganese and potassium digestibilities), the opposite was shown for carp (improved mineral retention including phosphorus, calcium and magnesium). Further while no effect on growth was seen in tilapia, a beneficial effect of oxalate was observed for carp. In mammals, oxalate may lead to hypocalcaemia, because dietary calcium may bind to oxalate and precipitate in the digestive tract leading to lower availability (Rahman et al., 2013). If the calcium / oxalate ratio in the diet is low, oxalate may be taken up in the blood stream and precipitate with calcium mainly in the kidney (Rahman et al., 2013). Continuous calcium oxalate formation in the kidney may compromise its functionality. Kidney stones in fish made of calcium oxalate have been observed before by Blazer & Wolke (1983). It is unfortunate that there was no histology conducted in Trial 5.1, which could maybe connect the higher calcium, magnesium and phosphorus retention in the high-oxalate diets to the formation of calcium oxalate stones. The original objective was to evaluate its effects on growth rather than pathogenesis, which is why no histology was considered in this first trial. Histology of Trial 5.2 showed calcareous structures in kidneys of tilapia, but not severe and with no differences between treatments.

The unexpected beneficial effects on growth are discussed in 5.1.4 and interpreted to be similar to other salts of organic acids altering the gut micro flora (Lueckstädt et al., 2008). It has been shown *in vitro* that oxalate has antimicrobial effects on a great variety of microorganisms (Borick & Bratt, 1961).

An anti-nutritional effect of oxalate concerning mineral digestibility could be shown for tilapia, however, without impact on growth. The binding of positively charged amino acids, much like what has been hypothesized to be an anti-nutritional effect of phytate (Spinelli et al., 1983), could not be observed for oxalate. The opposite effects of oxalate on mineral retention in carp and tilapia need further investigation, but may be associated to differences in digestive tract pH (Hua & Bureau, 2010).

Dietary oxalate showed an impact on body lipid metabolism on carp and tilapia. There was a tendency of lower blood plasma cholesterol in both species when fish were fed diets containing 1.5% or 2.5% oxalate. For carp, this was reflected in significantly lower body lipid content in these treatments, while in tilapia only treatment 2.5% was significantly lower. These results are interesting when trying to interpret why carp and tilapia showed lower body lipid content at all inclusion levels in trials where JKM was replacing fishmeal as the sole protein source (Trials 3.1 and 3.2). Minimum investigated dietary inclusion levels at 50% fishmeal replacement level were 33.8% (Trials 3.1) and 34.7% (Trial 3.2) of JKM, which at 2.44% oxalate content of JKM would yield 0.83% and 0.85% total dietary oxalate, respectively. The amounts of wheat flour included in these diets increases that content by only around 0.02% and can therefore be neglected as a dietary oxalate source (Siener et al., 2006). Nevertheless these amounts of oxalate may have influenced body lipid composition in these trials. In line with this, is the higher body lipid content observed in Trials 3.3 and 3.4. Here, maximum inclusion level of JKM were 16.2% and 14.0%, respectively, which results in 0.40% and 0.34% oxalate, too low to influence body lipid content according to the results of chapter 5.

6.5 Aims and scopes of the project

The aims and scopes of the project outlined in chapter 1.5 were covered as following:

1. Replacement level of fishmeal by JKM in diets for carp and tilapia

If fishmeal is given as the only protein source, the present work showed it to be equally replaceable by JKM only at 50% for carp and only if JKM was incubated with citric acid and phytase beforehand. For tilapia, 50% and 75% replacement resulted in slightly, but not significantly, slower growth than the control. Replacing 100% of fishmeal protein by JKM resulted in significantly slower growth than the control, but was not different to replacing 50% or 75% in tilapia and on an absolute scale still demonstrated good feed conversion.

2. JKM as a protein source in practical diets for carp and tilapia

Research including JKM in practical diets for carp and tilapia has not been conducted before. The present results showed that fishmeal could be replaced 100% by JKM in diets for carp when protein contribution from fishmeal was only 25%. Growth of the 100% replacement treatment was even significantly improved compared to the control if 0.5% of a sodium formate / formic acid mix was included.

For tilapia a negative linear correlation was revealed, however, 100% replacement levels still showed good specific growth rates above 3% / day and a feed conversion ratio of 1.10 ± 0.06 for the duration of the trial period.

3. The role of phytate and phytase in JKM based diets

Whether phytate reduces amino acid digestibility in fish remains unclear. Phytase addition does most likely not influence amino acid digestibility directly. Phytase supplementation to non-phosphorus supplemented diets slightly increased growth, most likely through phosphorus liberation from phytate. Phosphorus liberation from phytate *in vivo* in tilapia was not as efficient as *in vitro*. *In vitro* pre-incubation of JKM with citrate and phytase enhanced growth performance in carp.

4. Dietary oxalate as an anti-nutritional factor in carp and tilapia

Carp showed improvements in growth and mineral retention through the addition of oxalate. In tilapia, digestibility of calcium, potassium, manganese and zinc digestibility was compromised through oxalate. Growth was not influenced. Oxalate at higher inclusions reduced body lipid content and presumably blood plasma cholesterol in both species. With respect to JKM, oxalate requires more attention in future research.

6.6 References

Akinleye, A. O., Kumar, V., Makkar, H. P. S., Angulo-Escalante, M. A., & Becker, K. (2011). *Jatropha platyphylla* kernel meal as feed ingredient for Nile tilapia (*Oreochromis niloticus* L.): growth, nutrient utilization and blood parameters. *Journal of Animal Physiology and Animal Nutrition*, *96*(1), 1–11.

Ali, A. A., Velasquez, M. T., Hansen, C. T., Mohamed, A. I., & Bhathena, S. J. (2004). Effects of soybean isoflavones, probiotics, and their interactions on lipid metabolism and endocrine system in an animal model of obesity and diabetes. *The Journal of Nutritional Biochemistry*, *15*(10), 583–590.

Bechara, J. A., Roux, J. P., Ruiz Diaz, F. J., Flores Quintana, C. I., & Longoni de Meabe, C. A. (2005). The effect of dietary protein level on pond water quality and feed utilization efficiency of pacu *Piaractus mesopotamicus* (Holmberg, 1887). *Aquaculture Research*, *36*(6), 546–553.

Blazer, V. S., & Wolke, R. E. (1983). Ceroid deposition, retinal degeneration and renal calcium oxalate crystals in cultured clownfish, *Amphiprion ocellaris*. *Journal of Fish Diseases*, *6*, 365–376.

Borick, P. M., & Bratt, M. (1961). Antimicrobial activity of some higher amine salts of carboxylic acids. *Applied Microbiology*, *9*, 475–477.

Chen, W., Ai, Q., Mai, K., Xu, W., Liufu, Z., Zhang, W., & Cai, Y. (2011). Effects of dietary soybean saponins on feed intake, growth performance, digestibility and intestinal struc-

ture in juvenile Japanese flounder (*Paralichthys olivaceus*). *Aquaculture*, *318*(1-2), 95–100.

Couto, A., Kortner, T. M., Penn, M., Østby, G., Bakke, A. M., Krogdahl, Å., & Oliva-Teles, A. (2014). Saponins and phytosterols in diets for European sea bass (*Dicentrarchus labrax*) juveniles: effects on growth, intestinal morphology and physiology. *Aquaculture Nutrition*, Online Version.

Deng, J., Kong, L., An, Q., Bi, B., & Tao, L. (2011). Effect of dietary pH adjustment on the utilization of supplemental methionine and lysine by juvenile Common Carp, *Cyprinus carpio*. *Journal Of The World Aquaculture Society*, *42*(5), 696–704.

El-Saidy, D. M. S. D., & Gaber, M. M. A. (2003). Replacement of fish meal with a mixture of different plant protein sources in juvenile Nile tilapia, *Oreochromis niloticus* (L.) diets. *Aquaculture Research*, *34*(13), 1119–1127.

El-Saidy, D. M. S. D., & Saad, A. S. (2011). Effects of partial and complete replacement of soybean meal with cottonseed meal on growth, feed utilization and haematological indexes for mono-sex male Nile tilapia, *Oreochromis niloticus* (L.) fingerlings. *Aquaculture Research*, *42*(3), 351–359.

Gaylord, T., Barrows, F. T., Teague, A. M., Johansen, K. A., Overturf, K. E., & Shepherd, B. (2007). Supplementation of taurine and methionine to all-plant protein diets for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *269*(1-4), 514–524.

Hardy, R. W. (2010). Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Aquaculture Research*, *41*(5), 770–776.

Hasan, M. R., Macintosh, D. J., & Jauncey, K. (1997). Evaluation of some plant ingredients as dietary protein sources for common carp (*Cyprinus carpio* L.) fry. *Aquaculture*, *151*(1-4), 55–70.

Hua, K., & Bureau, D. P. (2010). Quantification of differences in digestibility of phosphorus among cyprinids, cichlids, and salmonids through a mathematical modelling approach. *Aquaculture*, *308*(3-4), 152–158.

Ireland, P. A., Dziedzic, S., & Kearsleyb, M. W. (1986). Saponin content of soya and some commercial soya products by means of high-performance liquid chromatography of the sapogenins. *Journal of the Science of Food and Agriculture*, *37*, 694–698.

Kumar, V., Makkar, H. P. S., & Becker, K. (2011a). Detoxified *Jatropha curcas* kernel meal as a dietary protein source: growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings. *Aquaculture Nutrition*, *17*(3), 313–326.

Kumar, V., Makkar, H. P. S., & Becker, K. (2011b). Evaluations of the nutritional value of *Jatropha curcas* protein isolate in common carp (*Cyprinus carpio* L.). *Journal of Animal Physiology and Animal Nutrition*, *96*(6), 1030–1043.

Kumar, V., Makkar, H. P. S., Devappa, R. K., & Becker, K. (2011c). Isolation of phytate from *Jatropha curcas* kernel meal and effects of isolated phytate on growth, digestive physiology and metabolic changes in Nile tilapia (*Oreochromis niloticus* L.). *Food and Chemical Toxicology*, *49*(9), 2144–2156.

Kumar, V., Akinleye, A. O., Makkar, H. P. S., Angulo-Escalante, M. A., & Becker, K. (2012). Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed on a diet containing *Jatropha platyphylla* kernel meal as a protein source. *Journal of Animal Physiology and Animal Nutrition*, *96*(1), 37–46.

Lee, K.-J., Rinchard, J., Dabrowski, K., Babiak, I., Ottobre, J. S., & Christensen, J. E. (2006). Long-term effects of dietary cottonseed meal on growth and reproductive performance of rainbow trout: Three-year study. *Animal Feed Science and Technology*, *126*(1-2), 93–106.

Liu, L. W., Su, J., & Luo, Y. (2011). Effect of partial replacement of dietary monocalcium phosphate with neutral phytase on growth performance and phosphorus digestibility in gibel carp, *Carassius auratus* gibelio (Bloch). *Aquaculture Research*, 1–10.

Lueckstädt, C. (2008). The use of acidifiers in fish nutrition. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *3*(44), 1–8.

Makkar, H. P. S., & Becker, K. (2009). *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. *European Journal of Lipid Science and Technology*, *111*(8), 773–787.

Makkar, H., & Becker, K. (2011). Detoxification of *Jatropha Curcas* meal for feeding to farm animal species and fish. *United States Patent Application Publication*.

Mazurkiewicz, J. (2009). Utilization of domestic plant components in diets for common carp *Cyprinus carpio* L. *Archives of Polish Fisheries*, *17*(1), 5–39.

Morales, G. A., Márquez, L., Saenz de Rodrigañez, M., Bermúdez, L., Robles, R., & Moyano, F. J. (2013). Effect of phytase supplementation of a plant-based diet on phosphorus and nitrogen bioavailability in sea bream *Sparus aurata*. *Aquaculture Nutrition*, *20*(2), 172–182.

Nwanna, L., Eisenreich, R., & Schwarz, F. (2007). Effect of wet-incubation of dietary plant feedstuffs with phytases on growth and mineral digestibility by common carp (*Cyprinus carpio* L). *Aquaculture*, *271*(1-4), 461–468.

Pfeffer, E., Beckmann-Toussaint, J., Henrichfreise, B., & Jansen, H. D. (1991). Effect of extrusion on efficiency of utilization of maize starch by rainbow trout. *Aquaculture*, *96*, 293–303.

Rahman, M. M., Abdullah, R. B., & Wan Khadijah, W. E. (2013). A review of oxalate poisoning in domestic animals: tolerance and performance aspects. *Journal of Animal Physiology and Animal Nutrition*, *97*(4), 605–614.

Siener, R., Hönow, R., Voss, S., Seidler, A., & Hesse, A. (2006). Oxalate content of cereals and cereal products. *Journal of Agricultural and Food Chemistry*, *54*(8), 3008–3011.

Spataru, P., Wohlfarth, G. W., & Hulata, G. (1983). Studies on the natural food of different fish species in intensively manured polyculture ponds. *Aquaculture*, *35*, 283–298.

Spinelli, J., Houle, C., & Wekell, J. (1983). The effect of phytates on the growth of rainbow trout (*Salmo gairdneri*) fed purified diets containing varying quantities of calcium and magnesium. *Aquaculture*, *30*(1-4), 71–83.

Xie, B. S., Zhu, X., Cui, Y., & Yang, Y. (2001). Utilization of several plant proteins by gibel carp (*Carassius auratus gibelio*). *Journal of Applied Ichthyology*, *17*(2), 70–76.