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Research Article

Montmorillonite Clay Minerals With or Without Microalgae as a Feed Additive in Larval White Leg Shrimp (*Litopenaeus vannamei*)

Harry W. Palm*, Hendrik Sörensen and Ulrich Knaus

Department of Aquaculture and Sea-Ranching, University of Rostock, Germany

***Corresponding author**

Harry W. Palm, Department of Aquaculture and Sea-Ranching, Faculty of Agricultural and Environmental Sciences, University of Rostock, Rostock, Mecklenburg-West Pomerania, Germany, Tel: 49 0 381 498 3730; Fax: 49 0 381 498 118 3730; E-mail: harry.palm@uni-rostock.de

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- *Litopenaeus vannamei*
- Mixed layer clay minerals
- Montmorillonite-illite/muscovite
- Survival
- Size distribution
- Microalgae

Abstract

Montmorillonite–illlite/muscovite mixed layer clay minerals with or without addition of *Chlorella vulgaris* microalgae powder were applied as a feed additive in white leg shrimp early stage PL 1 postlarvae (12 days post hatch), 2x *Litopenaeus vannamei*. Three independent experiments were conducted in four identical recirculation systems, each consisting of 4 aquaria with a water volume of 750 L and a flow through of 250% per hour. With a stocking density of 150 post larvae in each aquarium per 0.5 m^2, representing super intensive conditions, the 2% application of the Friedland clay mineral demonstrated a positive effect onto *L. vannamei* survival, final total weight gain and partly reduced the feed conversion ratio (FCR) during three independent trials. In contrast application of 1% clay mineral reduced the shrimp performance compared with the control. Size distribution of shrimp was reduced for the 2% clay or the 2% clay/2% algae treatment groups, resulting in a lower maximum weight difference and lower standard deviation compared with the 1% clay treatment group and the control. Taking into account the higher survival rates and the observation that weak shrimps where directly consumed by the other animals in the aquaria, the clay mineral feed additive might have suppressed cannibalism and resulting in a more even size distribution and a healthier stock. The applied *Chlorella vulgaris* microalgae originated from a bioreactor attached to a power station fed with CO_2, making a direct use of the microalgae products in combination with montmorillonite-illlite/muscovite mixed layer clay minerals as a feed additive for healthy aquaculture feeds possible.

INTRODUCTION

Brackish water shrimps belonging to the family Penaeidae are a highly valuable food commodity. Although shrimps contributed only 6.5% to the total world aquaculture production by quantity, they represented 14% of the value of aquaculture products in 2012. An increasing demand, limited production volumes and rising shrimp prices are responsible for this trend [1]. One of the main obstacles in shrimp production is disease problems, which regularly lead to higher mortality rates and production losses. Measures of disease control, stabilization of the immune system and improving the health status of the shrimps have been focused in former investigations [2-6].

The reduction of antibiotics and other chemicals used in shrimp production is important for an environmentally sustainable development of the global aquaculture [4,7-11]. Different feed additives, bioactive components and probiotics have been reported to support shrimp health and growth [12-15] and have been discussed as an alternative for antibiotic use [3]. Feed additives like vitamins, minerals or plant compounds are well known for their positive effects and commonly used in shrimp aquaculture [15-17]. Especially algae and their compounds as feed ingredients have been suggested to support shrimp production [18-23].

Clay minerals have been demonstrated to have positive effects onto animal and even human health [24-27]. As an animal feed additive they bind and eliminate mycotoxins [28], improve weight gain, feed efficiency and egg production in poultry farming [29-31], and increase the average daily weight gain and performance in pig [32-35] and sheep farming [36]. Earlier studies suggest that the use of clay minerals might have beneficial effects during cultivation of aquatic organisms [37,38]. Clay was

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used to increase turbidity and to reduce the bacteria level in the production water of cod larvae with indirect positive effects onto growth and survival [39]. Direct positive effects of different clay minerals as a feed additive onto growth and feed conversion in salmonid fish aquaculture were documented [40].

Friedland montmorillonite–illite/muscovite alternating sequence clay minerals have been reported being a highly efficient absorbent of mycotoxin from animal feed. 100% of Aflatoxin B1 and Fumonisin, 80% of Ochratoxin and Zearalenone and 60% of T2-Toxin were absorbed during experimental investigations [41]. A significant reduction of inflammatory bowel diseases was reported by [26] through in situ perfusion of an extract of this clay mineral with rats. Friedland clay minerals are a highly efficient phosphate binder and assumed to have positive effects onto human kidney diseases [27]. As a feed additive, this specific clay mineral increased the body weight and feed intake in pig farming, milk production and feed intake in cow farming, and induced a significant increase in body weight gain and feed intake in chicken farming [42-44]. So far, clay minerals have not been applied in shrimp aquaculture, though these animals are highly intolerant to mycotoxins [45,46]. *L. vannamei* is surrounded by different clay minerals in the natural habitat, with a possible effect onto infectious shrimp disease agents. The purpose of the present study is to apply Friedland clay minerals as a feed additive for shrimp culture, with and without addition of the green algae *Chlorella vulgaris*. Possible reasons for the reported effects onto shrimp mortality, total biomass, growth performance and size range are discussed.

MATERIALS AND METHODS

Experimental design

The experiments were conducted in four identical recirculation systems, each with a water volume of 750 L and a flow through of 250% per hour. Each system consisted of 4 glass aquaria (100 cm x 50 cm x 50 cm) with a volume of approx. 165 L each combined with a filter unit including a biofilter, protein skimmer, sump and pipes (90 L). For each experiment, early stage larval White-Leg-Shrimps (*Litopenaeus vannamei*) were obtained as PL 1postlarvae (12 days post hatch) from Shrimp Improvement Systems LLC (SIS), 88081 Overseas Highway, Islamorada, FL 33036, USA. Before the experiment the shrimp larvae were acclimatized to the experimental conditions in a special 500 L acclimatization tank, and fed first with living Artemia for 2 days, and weaned with Artemia combined with the reference feed for 7-10 days.

During the feeding experiments all systems were filled with purified water, salted to a salinity of 13-15‰, and all tanks were randomly stocked with 100-150 PL 1 White-Leg-Shrimps (*Litopenaeus vannamei*). The water quality was monitored using Hach-Lange spectral photometer DR 3900 and Hach HQ40d portable meter (Hach Lange GmbH, Germany), and maintained in the optimum range (temperature 29-31 °C, salinity 13-16‰, pH 7.7-8.0, dissolved oxygen 6.7-8.0 mg L^{-1}, total NH_3 0.01-0.07 mg L^{-1}, nitrite 0.01-0.11 mg L^{-1}, and nitrate 2.0-16.6 mg L^{-1}) during the run of the experiments. The feed amount for each aquarium was calculated daily according to the biomass of the shrimp (5-15% measured) during Experiment I, and kept unchanged during a prestudy and Experiments II and III. The reference diets were fed by automatic feeders 6-8 times a day.

Experiments (prestudy, I-III)

A prestudy with stocking densities of 50, 100, 150 and 200 PL 1 in each system with the use of the commercial feed Le Goussant was carried out for 60 days, monitoring survival rates and total biomass in four systems. During Experiment I the aquaria in each of the four systems were stocked with 150 post larvae (equals 300 PL m^{-2}). As a control standard the commercial feed from the prestudy was used. The same feed was mixed with the feed additive of 1% clay, 2% clay and a mixture of 1.5% clay and 0.5% algae paste (*Chlorella vulgaris*) originally obtained as a dilution from an algae bioreactor. This trial was carried out for 60 days, and the feeding was adapted to the weekly-calculated biomass in each tank. The diets were formulated at IGV GmbH, Getreidemittelverarbeitung, Potsdam (Germany). During feed preparation, the new feed mixture was heated again above 90 °C.

For Experiment II each aquarium was stocked with 100 post larvae. The feeding was kept unchanged for the entire 44 days. Four different diets from the same raw material, with the clay mineral/algae additive added, were formulated at Research Diet Services BV, WijkbijDuurstede (the Netherlands), each in 3 different pellet sizes (300-500 µm; 500-700 µm; 700-1400 µm). The reference diet contained fish meal (40.3%), shrimp meal (10%), squid meal (6%), wheat (13.53%), wheat flour (25%), fish oil (2%), vitamin premix (1.5%), Lecithin (1%), salt (0.6%) and Cholesterol (0.07%). The three test diets were produced including 1% clay additive, 2% clay additive and 2% clay powder combined with 2%microalgae meal (*Chlorella vulgaris*). The test diets were analyzed according to their feed composition and energy contents (Table 1).

Experiment III repeated Experiment II by testing the control diet, 2% clay powder and 4% of the clay additive (2%)/microalgae (2%) mixture for 32 days, using 150 post larvae of a new shrimp batch and a constant feeding regime. As a major difference, the new control group was kept in the best performing recirculation system from Experiment II (50:50 clay powder and microalgae mixture) and vice versa, excluding an experimental set up bias.

Clay powder and microalgae

The clay mineral was mined in Friedland in Mecklenburg-West Pomerania (Germany) by FIM Biotech GmbH. It is registered as "FIMIX (100% Friedland Clay Mineral) under EG (VO) 1060/2013",currently registered as a montmorillonite-illite mixed layer clay mineral, with a positive EFSA opinion concerning product security and running efficacy tests. The Friedland clay minerals were formed in shallow marine environments as a result of sedimentation of volcanic tephra and eroded detrital material, and underwent early diagenesis. Due to complex formation history, the Montmorillonite Friedland clay minerals contain, among others, mixed-layered illite and muscovite, as well as kaolin [47]. It has a less montmorillonite content (40-60%) compared with the bentonites (60-80%). Furthermore, it differs in its composition from all other clay mineral sources [48] with a mineral fingerprint, according to the following formula $(Na_{0.58}Ca_{0.03}Mg_{0.10}Al_{2.22}Fe^{3+}{}_{1.09}Fe^{2+}{}_{0.18}Mg_{0.59}Si_{7.80}Al_{0.20}O_{20}(OH)_4)$ [49]. It consists of SiO_2 (57.9-59.5 % total weight, Al_2O_3(17.0-18.5%),

Table 1: Nutrient and fatty acid content of diets from Exp II & III.

	Control	1% MMT[1]	2% MMT[1]	Mixture[2]
Nutrients	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**
Moisture (%)	5.4	5.8	6	8
Ash (%)	10.3	11.4	12.1	12.4
Protein (%)	40.8	40.6	40.4	39.2
Crudefiber (%)	2.3	2.3	2.5	2.3
Fat (%)	7.6	7.3	7.6	7.3
Fatty acid (%)				
C14:0	0.00	0.00	6.65	6.17
C16:0	23.84	23.94	22.11	22.65
C16:1	6.97	8.74	7.65	7.34
C18:0	4.83	4.61	5.08	4.25
C 18:1 n9c	19.25	18.79	18.01	17.67
C 18:2 n6c	17.85	17.05	16.14	16.45
C 20:1	2.84	2.7	2.58	2.53
C 18:3 n3	2.84	2.57	2.02	1.88
C 20:2	2.85	1.83	1.9	1.91
C 22:1 n9	0.00	1.92	0.00	0.00
C 23:0	0.00	0.00	0.81	0.79
C 22:2	0.00	0.00	0.00	0.91
C 20:5 n3	8.62	8.45	8.03	8.1
C 22:6 n3	10.11	9.4	9.02	9.35
Energy (kJ kg^{-1})	17,765.9	17,468.5	17,290.4	16,459.7
Energy (%)	100	98.3	97.3	92.7

Abbreviations: Values are based on the analysis at LUFA, Rostock (Germany)[1], MMT = Montmorillonite[2], Mixture = for Exp II & III the clay/algae mixture contained 2% clay and 2% algae powder.

Fe_2O_3(5.9-7.0%), K_2O (2.8-3.5%), MgO (1.5-2.6%), Na_2O (0.9-1.5 %), TiO_2(0.6-1.5), CaO (0.25-0.35), P_2O_5(0.09-0.15), and others (8.9-10.5%), or 23% Si, 9.6% Al, 3.8% Fe, 0.9% Mg, 0.2% Ca, 0.7% Na, 1.7% K, and 0.5% Ti. Other characteristics are the particle size of 90% ≤ 80 µm or 50% ≤ 25 µm, pH of 7.3, conductivity of 346 µS, cation exchange capacity of 38 meq/100 g, and exchangeable cations of Ca, K, Mg, Na.

The microalgae *Chlorella vulgaris* originated form a photo-bioreactor at a power station of the city Senftenberg (Vattenfall GmbH, Germany). FIM Biotech GmbH has developed a low-cost process, directly binding harvested *Chlorella vulgaris* dilution by using a defined Friedländer clay mineral, resulting in ready-to-add mineral/algae composite as a feed additive. The protein-rich biomass, in our case produced from CO_2-containing flue gases from a conventional power plant, resulted from sedimentation under the presence of magnesium, followed by a drying and downsizing process. The clay/algae mixture was suitable as a shrimp feed additive, with the clay mineral already approved in Europe as a feed additive material [41].

Data collection and statistical analyses

At the beginning of each experiment, initial weights of the shrimps were taken. During the run of the experiments, random samples of 35 individuals from each aquarium were measured every two weeks. At the end of each experiment all shrimps were weighed and measured. Weight gain, specific growth rate (SGR, percentage weight gain per day), the feed conversion ratio (FCR) and survival were calculated for each group of shrimps.

The data were analyzed by using One-Way ANOVA. Differences of means were evaluated for significance by the range test of TUKEY-HSD ($p<0.05$) for homogeneous variances and by the DUNNETT-T3 ($p<0.05$) test for inhomogeneous variances respectively. Calculations were performed with the SPSS 20.0 software package [42].

RESULTS

The optimal stocking density for the system used in this study was determined from the prestudy. The best survival rates were observed at stocking densities of 100-150 shrimps per tank or 200-300 post larvae per m^2.

Experiment I

The results for this 60 days feeding trial are given in Table 2. During this experiment, the mean initial body weight of 0.047 g increased to 2.22-2.65 g. Best growth (final weight) was observed with the control (2.65 g), whereas treatment 1%, 2% and the mixture diet showed no significant final weights from 2.22-2.32 g. Total weight gain was best in the control group with 265.43 g (100%), and not significant with the 2% treatment with 261.69 g (98.6%, Figure 1). Furthermore, for all other growth parameters, the control group achieved the best results (the treatment feed had additional feed processing compared with the control). The final biomass and total weight gain were significant higher than in the 1%-clay mineral treatment and higher than in the clay/algae mixture treatment. The treatment with 2% montmorillonite showed no significant differences with the control group. The SGR in the 2%-treatment-group was lower than in the other 3 groups, with 6.43% d^{-1} growth per day compared to 6.73% d^{-1} in the control group, 6.50% d^{-1} in the 1% treatment group and 6.47% d^{-1} in the mixture treatment group. The lowest FCR was also observed in the control group (1.07), ranging between 1.2-1.3 in the three tested groups.

In contrast to the respective growth parameters, highest survival was observed in the 2% clay mineral treatment-group (79.75%), although there were no significant differences to the control group (68.10%) and to the clay/algae-group (68.75%, Figure 2). The 1% treatment-group achieved the lowest survival (56.25%). The physical and chemical water parameters showed no significant differences among the feeding groups during this experiment except salinity with significant slightly differences between all groups (Table 3).

The size distribution of the final shrimp weight differed between the different treatment groups in all three experiments (Figs. 3-5). In experiment I the median, minimum and maximum weight (g) in the control, 1% clay, 2% clay and 2% algae/2% clay treatment groups was 2.68 (0.13, 5.50), 2.36 (0.4, 4.2), 2.28 (0.24, 4.04), and 2.26 (0.75, 4.30), respectively. The most even size distribution was observed for the 2% algae/2% clay treatment group (Figure 3), resulting in a maximum weight difference of 5.37 g (±0.84) for the control, compared with 3.8 g (±0.7) in the

Table 2: Shrimp growth and survival parameters during 3 different feeding experiments, means (± SD), groups with not sharing a superscript in the same column are significantly different ($p<0.05$, n=4), for Experiment I, mixture-treatment, the clay/algae mixture contained 1.5% clay and 0.5% algae powder, for Experiment II and III the clay/algae mixture contained 2% clay and 2% algae powder.

EXP I						
group	**survival [%]**	**final weight [g]**	**final biomass [g]**	**total weight gain [g]**	**SGR^1 [% d^{-1}]**	**FCR^2**
Control	68.10^{ab}±9.43	2.65^{a}±0.84	270.34^{a}±25.14	265.43^{a}±25.79(100%)	6.73^{a}±0.10	1.07^{a}±0.06
1%-Treatment	56.25^{a}±9.11	2.32^{b}±0.70	196.24^{b}±23.34	190.38^{b}±23.70(74.2%)	6.50^{ab}±0.06	1.34^{b}±0.12
2%-Treatment	79.75^{b}±5.91	2.22^{b}±0.61	265.64^{a}±6.80	261.69^{a}±9.40(98.6%)	6.43^{b}±0.10	1.20^{ab}±0.06
Mixture-Treatment	68.75^{ab}±11.00	2.29^{b}±0.60	234.83^{ab}±23.95	231.67^{ab}±25.54(87.3%)	6.47^{ab}±0.22	1.23^{ab}±0.10
EXP II						
group	**survival [%]**	**final weight [g]**	**final biomass [g]**	**total weight gain [g]**	**SGR^1 [% d^{-1}]**	**FCR^2**
Control	33.00^{a}±10.61	2.85^{a}±1.66	67.60^{a}±19.10	62.50^{a}±19.10 (100%)	5.79^{ab}±0.76	1.15^{ab}±0.13
1%-Treatment	16.25^{a}±5.85	3.23^{a}±2.20	32.34^{a}±26.09	27.24^{a}±26.09(43.7%)	3.75^{a}±1.56	2.09^{b}±1.03
2%-Treatment	32.00^{a}±11.05	3.34^{a}±1.29	73.45^{a}±33.70	68.35^{a}±33.70(110%)	5.89^{b}±1.00	1.02^{ab}±0.23
Mixture-Treatment	54.75^{b}±7.80	3.18^{a}±1.15	151.25^{b}±4.67	146.15^{b}±4.67 (234%)	7.70^{b}±0.07	0.76^{a}±0.01
EXP III						
group	**survival [%]**	**final weight [g]**	**final biomass [g]**	**total weight gain [g]**	**SGR^1 [% d^{-1}]**	**FCR^2**
Control	26.00^{a}±10.46	1.14^{a}±0.48	29.54^{a}±8.10	27.58^{a}±8.10(100%)	14.08^{a}±0.48	1.37^{a}±0.43
2%-Treatment	41.50^{a}±15.86	0.95^{b}±0.38	39.37^{a}±7.45	37.41^{a}±7.45 (135.5%)	13.55^{a}±0.65	0.93^{a}±0.16
Mixture-Treatment	45.00^{a}±9.90	0.89^{b}±0.36	40.15^{a}±5.81	38.19^{a}±5.81(138.4%)	13.25^{a}±0.38	0.94^{a}±0.17

Abbreviations: ^{1}SGR = Specific growth rate [% d^{-1}] = (ln final weight-ln initial weight) x t^{-1} x 100; t = 60 days for Exp I, 44 days for Exp II and 32 days for Exp III,^{2} FCR = Feed conversion ratio = feed consumed [g] x weight gain [g]$^{-1}$.

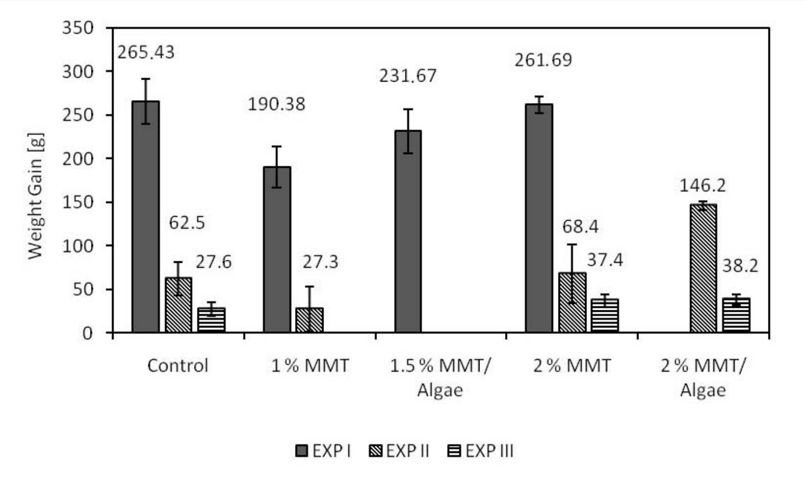

Figure 1 Weight gain [g] of *L. vannamei* during Experiments I-III.

1% clay treatment group, 3.8 g (±0.61) in the 2% clay treatment group, and 3.55 g (±0.60) in the mixture treatment group.

Experiment II

Experiment II was carried out for 44 days and results are given in Table 2. During the acclimatization phase in a separate holding tank, we recorded a problem with the biofilter resulting in ammonia (0.8 mg L^{-1}) and nitrite (0.135 mg L^{-1}) peaks, with possible negative effects onto shrimp post larvae survival in the following cultivation in the recirculation units. During the run of the experiment, water parameters were stable, with only slightly differences between the four treatment groups (Table 3).

Compared with Experiment I, the shrimp larvae had a significantly lower survival rate (Table 2). Comparing the four treatment groups, the clay/algae-treatment-group had a significant higher survival rate (54.75%) than those shrimps fed with the control diet (33.00%). The 1%-clay mineral treatment (16.25%) was lower, and the 2% clay mineral treatment (32.00%) group showed no significant better survival to the control group. All treatment groups (3.18-3.34 g) achieved a higher mean final weight than the control group (2.85 g), even though there were no significant differences. The shrimps fed the clay/algae mixture treatment diet had a significantly higher final biomass (151.25 g) and weight gain (234%) compared with the control group

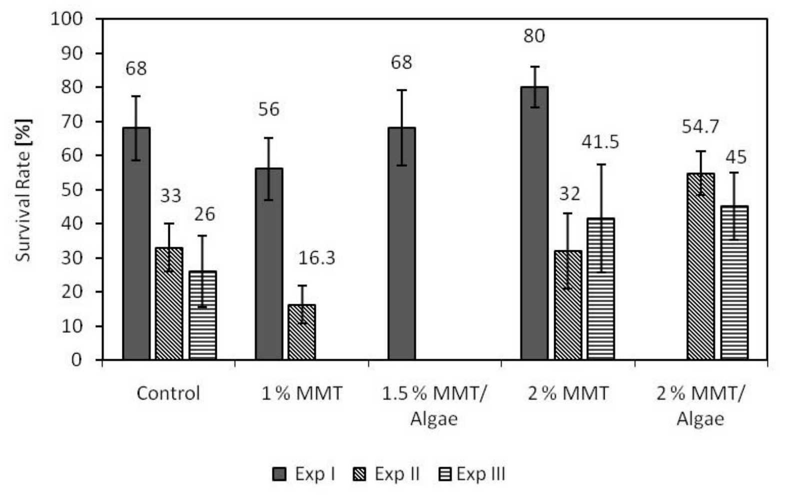

Figure 2 Survival [%] of *L. vannamei*during Experiments I-III.

Table 3: Water parameters during Experiments I-III, means (± SD). Groups with not sharing a superscript in the same column are significantly different ($p<0.05$, $n=4$).

EXP I							
Parameter	**Oxygen**	**pH**	**Temperature**	**Salinity**	**Ammonia**	**Nitrite**	**Nitrate**
	[mg L^{-1}]		**[°C]**	**[‰]**	**[mg L^{-1}]**	**[mg L^{-1}]**	**[mg L^{-1}]**
Control	$6.75^{a}\pm0.24$	$7.86^{a}\pm0.46$	$30.69^{a}\pm0.76$	$13.90^{a}\pm0.18$	$0.05^{a}\pm0.01$	$0.10^{a}\pm0.08$	$15.23^{a}\pm7.70$
1% Clay	$6.91^{a}\pm0.22$	$8.09^{a}\pm0.39$	$31.23^{a}\pm0.56$	$13.63^{b}\pm0.26$	$0.05^{a}\pm0.02$	$0.10^{a}\pm0.07$	$14.81^{a}\pm6.75$
2% Clay	$6.78^{a}\pm0.37$	$7.75^{a}\pm0.41$	$31.08^{a}\pm0.67$	$14.18^{c}\pm0.31$	$0.05^{a}\pm0.03$	$0.10^{a}\pm0.05$	$14.70^{a}\pm5.78$
Mixture	$6.95^{a}\pm1.07$	$7.84^{a}\pm0.41$	$31.12^{a}\pm0.57$	$14.83^{d}\pm0.15$	$0.06^{a}\pm0.03$	$0.11^{a}\pm0.08$	$16.65^{a}\pm8.63$
EXP II							
Parameter	**Oxygen**	**pH**	**Temperature**	**Salinity**	**Ammonia**	**Nitrite**	**Nitrate**
	[mg L^{-1}]		**[°C]**	**[‰]**	**[mg L^{-1}]**	**[mg L^{-1}]**	**[mg L^{-1}]**
Control	$7.80^{a}\pm0.20$	$7.82^{a}\pm0.09$	$30.34^{a}\pm0.42$	$13.70^{a}\pm0.35$	$0.07^{a}\pm0.02$	$0.02^{a}\pm0.01$	$2.94^{a}\pm0.85$
1% Clay	$7.70^{ab}\pm0.19$	$7.95^{b}\pm0.05$	$30.77^{a}\pm0.61$	$13.78^{a}\pm0.19$	$0.05^{b}\pm0.02$	$0.02^{a}\pm0.01$	$2.53^{a}\pm0.44$
2% Clay	$7.61^{ab}\pm0.17$	$7.91^{ab}\pm0.07$	$30.86^{a}\pm0.50$	$14.08^{ab}\pm0.27$	$0.05^{b}\pm0.01$	$0.02^{a}\pm0.00$	$3.03^{a}\pm1.68$
Mixture	$7.52^{b}\pm0.26$	$7.84^{ab}\pm0.18$	$30.72^{a}\pm0.42$	$14.33^{b}\pm0.06$	$0.06^{ab}\pm0.02$	$0.02^{a}\pm0.01$	$3.20^{a}\pm1.21$
EXP III							
Parameter	**Oxygen**	**pH**	**Temperature**	**Salinity**	**Ammonia**	**Nitrite**	**Nitrate**
	[mg L^{-1}]		**[°C]**	**[‰]**	**[mg L^{-1}]**	**[mg L^{-1}]**	**[mg L^{-1}]**
Control	$7.75^{a}\pm0.13$	$7.96^{a}\pm0.14$	$30.64^{a}\pm0.85$	$14.67^{a}\pm0.12$	$0.01^{a}\pm0.01$	$0.01^{a}\pm0.00$	$2.42^{a}\pm0.79$
2% Clay	$8.04^{b}\pm0.16$	$7.76^{b}\pm0.19$	$29.91^{a}\pm1.01$	$15.37^{b}\pm0.16$	$0.01^{a}\pm0.01$	$0.01^{a}\pm0.00$	$2.08^{a}\pm0.89$
Mixture	$7.74^{a}\pm0.15$	$7.92^{ab}\pm0.14$	$30.23^{a}\pm0.74$	$14.59^{a}\pm0.13$	$0.03^{a}\pm0.03$	$0.01^{a}\pm0.00$	$2.22^{a}\pm0.97$

(100%). Shrimp larvae fed with the 2% clay mineral treatment diet had a slightly higher final biomass (73.45 g) and weight gain (110%) than the control group and the 1% treatment group (32.34 g; 43.7%), but without any significant differences. This was in contrast to the feed energy content, that was highest in the control feed compared with the 1% clay (-1.7%), 2% clay (-2.7%) and the 2% clay/2% algae (-7.3%) treatments (Table 1). Taking into account the energy contents, the adjusted biomass gain based on energy input was 113% (2% clay mineral), 44.5% (1% clay mineral) and 251% (2% clay/2% algae) compared with the control.

The size distribution of the final shrimp weight differed between the different treatment groups also at the end of Experiment II (Figure 4). The median, minimum and maximum weight in the control, 1% clay, 2% clay and 2% algae/2% clay treatment groups was 2.43 (0.14, 8.43), 2.96 (0.18, 8.43), 3.19 (0.67, 6.21), and 3.31 (0.14, 6.21), respectively. The most even

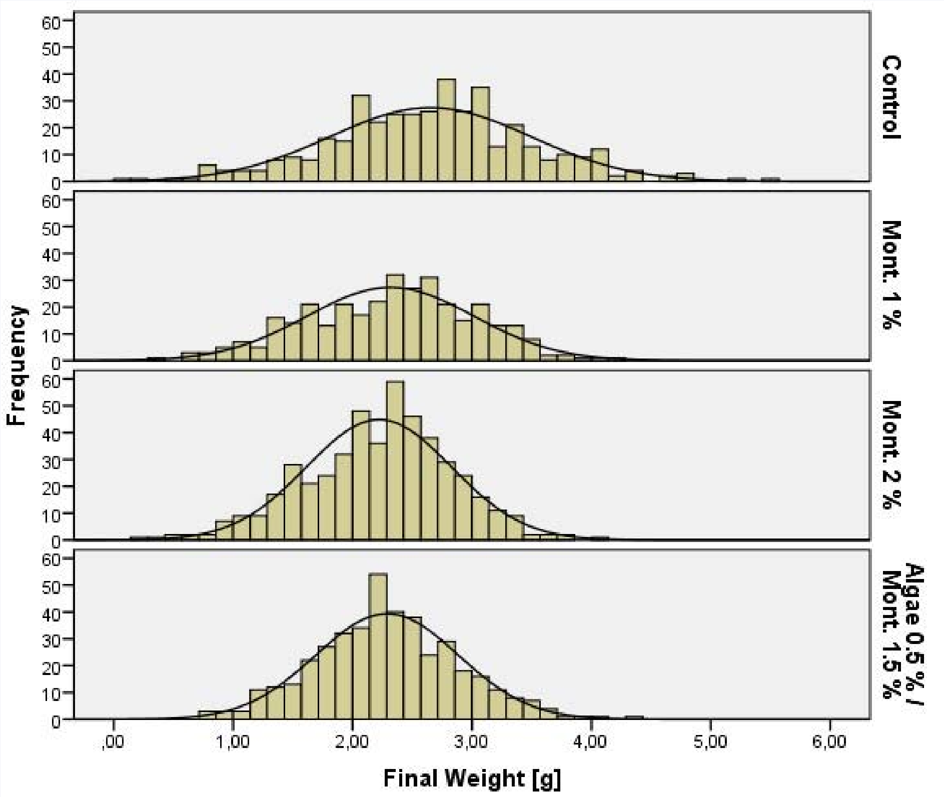

Figure 3 Size distribution histogram of shrimp final weight [g] and different treatment groups of clay in Experiment I.

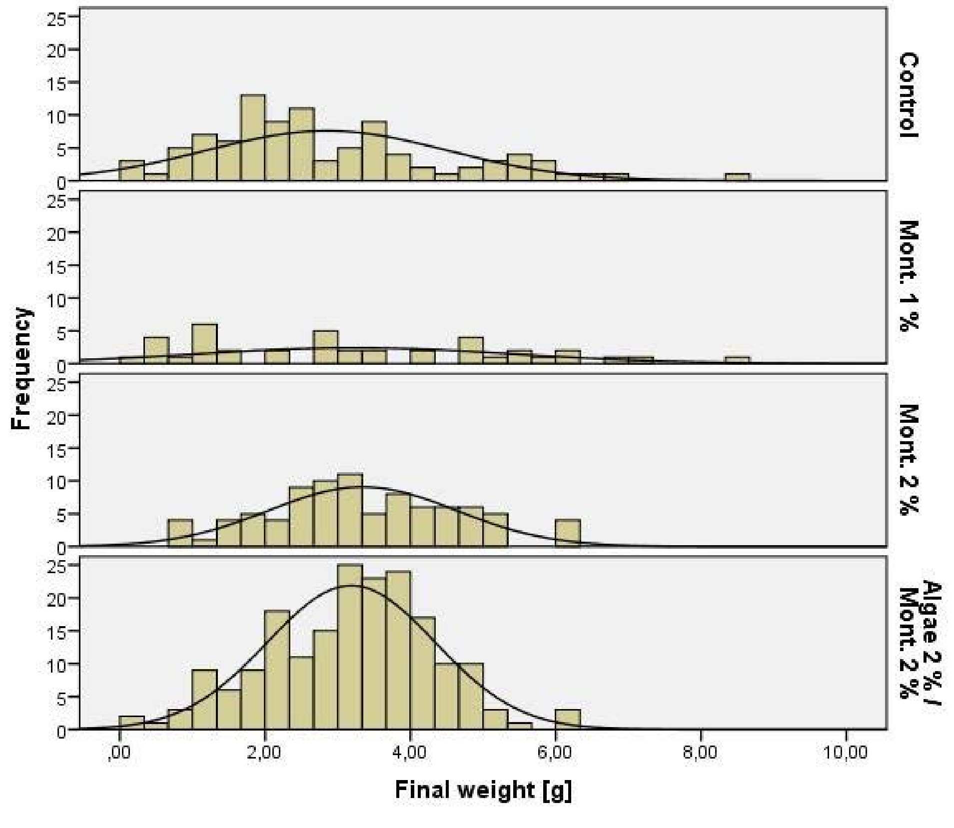

Figure 4 Size distribution histogram of shrimp final weight [g] and different treatment groups of clay in Experiment II.

size distribution was observed for the 2% clay treatment group, resulting in a maximum weight difference of 5.54 g (±1.29), followed by the 2% algae/2% clay (6.07, ±1.15), 1% clay (8.25, ±2.20) treatment group and the control (8.29, ±1.66, Figure 4).

Experiment III

The results of Experiment III showed significant difference only between the control treatment groups with a greater final weight of 1.14 g compared to the other diets (Table 2). However, the 2% clay mineral treatment (41.50%) and the clay/algae mixture treatment (45.00%) groups had a higher survival rate than the control group (26.00%). As a result of the higher survival rate and shrimp number in the aquaria, the same feed intake and slightly reduced energy content, the mean final weight of the shrimps was lower in both treatment groups than in the control. However, weight gain (surviving shrimps x individual final weight) was 30-35% higher in both treatment groups compared with the control.

The SGR slightly better results in the control group compared to the 2% treatment and the clay/algae mixture (Table 2). The control had a higher FCR of 1.37 compared with a FCR of 0.93 for the 2% treatment group and 0.94 for mixture treatment group.

The size distribution of the final shrimp weight differed between the three treatment groups in Experiment III (Figure 5). The median, minimum and maximum weight in the control, 2% clay and 2% algae/2% clay treatment groups was 1.01 (0.44, 3.20), 0.87 (0.19, 2.12), and 0.84 (0.22, 2.60), respectively. The most even size distribution was observed for the 2% clay treatment group (Figure 5), resulting in a maximum weight difference of 1.93 g (±0.38), compared with in the other treatment group (2.36 g, ±0.36) and the control (2.76 g, ±0.47).

DISCUSSION

Clay minerals are commonly used as a feed ingredient in land based animal husbandry, e.g. chicken-, pig- or cow- farming. As a feed additive, they have been demonstrated to have positive effects onto the treated animals, e.g. clay minerals can improve weight gain, feed efficiency and egg production in poultry farming. A significant increase in average weight gain was observed by adding 1-3% bentonite to the feed [30]. Similar results under 1-2% treatments with sodium bentonite were found by [31]. They monitored a higher feed intake and weight gain and lower FCR in the treated animals. Other studies noticed an increase in average daily weight gain and growth performance in pig farming. A slight increase in feed intake, weight gain and feed conversion for growing pigs by the use of 0.15% montmorillonite was reported by [34]. In a second study, they repeated these results for weanling pigs by using 0.2% montmorillonite [35]. This challenges the application of these feed additives also for fish and shrimp aquaculture.

In the cultivation of aquatic animals clay was used to increase turbidity and to reduce the bacteria level in the production water of cod larvae, and observed indirect positive effects onto growth and survival [39]. Under the clay treatment the larvae had a slightly higher dry weight and a 20-25% better survival.

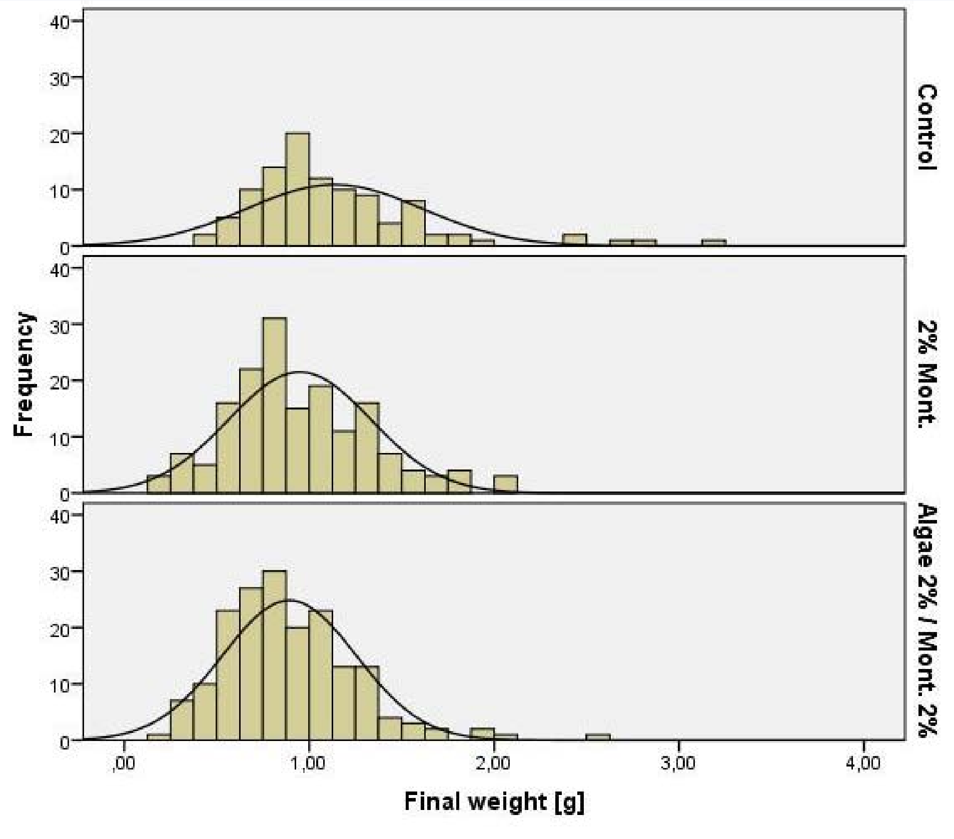

Figure 5 Size distribution histogram of shrimp final weight [g] and different treatment groups of clay in Experiment III.

The application of clay minerals in rainbow trout aquaculture was studied with direct positive effects onto growth and feed conversion [40]. The major outcome of his study was that the efficiency of the additive depended on the source of the clay mineral as well as the given amount, resulting in an increase in weight gain of 10% at a clay content in the trout feed of 2 and 5%. This is in the range of the results in the present study, where the Friedland clay mineral demonstrated a positive effect onto *Litopenaeus vannamei* survival, final total weight (110%) and FCR at 2% (with or without algae additive) in three independent trials (Table 2). On the other hand, the application of 1% clay mineral reduced the shrimp performance in the present trials compared with the control. Furthermore, the effects of montmorillonite clay minerals were analyzed onto Nile tilapia (*Oreochromis niloticus*) growth with the use of 0.15% montmorillonite in the fish diets [37]. A slight enhancement of growth performance and survival was observed. According to our results, this might be caused by an underestimate of the needed amount (0.15 vs. 2%) or the kind of clay mineral that was used.

Clay minerals have many medical and therapeutically effects onto animal and human health [24,25,51], due to desirable physical and physico-chemical properties, such as high adsorption capacity, specific surface area, swelling capacity, and reactivity to acids [24,52]. They are used in the pharmaceutical industry in many different forms, and administered either orally or topically e.g. as antiseptics, disinfectants, and even anti-inflammatories [25]. Montmorillonite has been demonstrated to have positive effects onto the intestinal microflora and antibacterial activities [53,54]. The influence of montmorillonite on the intestinal microflora, digestibility and digestive enzyme activities in the Nile Tilapia, *Oreochromis niloticus* was studied by [38]. They recorded an improvement of growth performance, reduction of intestinal aerobic bacterial counts and effects onto the composition of the intestinal microflora. Furthermore, they recorded an improvement of the digestibility of dry matter and crude protein, and improved enzyme activities of total protease, amylase, lipase and alkaline phosphatase. The antibacterial activity of the montmorillonite resulted in positive enzyme activities, promoting digestion and adsorption of dietary nutrition [38]. The specially characterized Friedland montmorillonite–illite/muscovite mixed layer clay mineral used in the present study has similar production and health promoting effects in animal husbandry. As a feed additive, this specific clay mineral, already known as a highly efficient mycotoxin binder, increased body weight and feed intake in pig farming, milk production and feed intake in cow farming, and induced a significant increase in body weight gain (approx. 10%) and feed intake in chicken farming [43-45]. A significant reduction of inflammatory bowel diseases was reported through in situ perfusion of an extract of this clay mineral with rats [26]. According to [27], Friedland clay minerals are a highly efficient phosphate binder, and assumed to have positive effects onto human kidney diseases. The use of clay minerals was reported to reduce Ammonium from wastewater [55]. The present study demonstrates, based on higher survival rates, such positive effects of this clay mineral also onto an invertebrate aquaculture species, and under recirculation aquaculture and experimental conditions. Possible negative effects such as nutrient binding capacities have not been tested so far.

We could observe significant different growth performance, survival rates, SGR, FCR and final size distribution of the tested shrimps under Friedland clay mineral application. The initiate stocking density with 200-300 post larvae m^{-2} was high, the glass aquaria without major hiding possibilities and a high ammonium load at the beginning of the second experiment represented non-favorable conditions for the shrimps during the run of the experiments. Experiment I showed better survival (79.75%) but less weight gain (262 g) in the 2% montmorillonite treatment group, compared with 68% and 265 g in the control. This is in contrast to the treatment of the test diets that were heated above 90 °C compared with the control diet, possibly affecting its nutritional value. Consequently, the control diet without feed additive and higher energy content should have performed better than the test diets including the feed additive. This is a good explanation for the lower final weight and the higher FCR observed for the three test groups. The total final weight gain of the 2% treatment group is caused by the highest survival rate during this experiment.

This results of the first experiment is supported by the other two experiments, where the water conditions where non-favorable (Experiment II) for the post larvae survival. Shrimps with the feed additive treatment demonstrated significant better survival, though the energy content of the feed was reduced between 1.7 and 7.3%. Especially after the high ammonium values during the acclimatization phase, shrimps treated with the clay/algae feed reached 234% of the weight gain compared with the control group, and an obvious lower FCR. This was repeated in Experiment III, with the best growth performance under treatment with 2% montmorillonite with or without microalgae powder. Though the three independent experiments achieved different shrimp performances, based on a different feed treatment before the experiments and water quality during the weaning phase, the same trend was observed. We can conclude that under three different scenario using different imported charges of post larvae, survival rates and total weight gain increased under treatment of 2% Friedland montmorillonite. On the other hand, the 1% feed additive showed a lower performance compared with the control, underlining the statement by [40] that not only the origin but also the concentration effects the growth performance in rainbow trout. Also in aquatic invertebrates, digestibility of nutrients might be supported through the clay feed additive, as described by earlier authors [37,38,53] and also for the higher vertebrates [34,35,43-45].

An important observation is the reduced size range of the clay mineral treatment groups after the three independent experiments, where the 2% clay additive performed best, resulting in the most uniform size distribution of the shrimps (Figures. 3-5). During experiment II, the 2% clay/2 % algae feed additive performed best while the 2% clay had a more similar size range after Experiments I and III. Taking into account the higher survival rates and the observation that weak shrimps where directly consumed by the other animals in the aquaria, less cannibalism might be the reason for this result. High stocking densities of shrimps cause increasing interactions between the single shrimp individuals, resulting in cannibalism and increasing mortality rates [56-58]. In our experiments, the stocking densities of 200-300 Shrimps per m^2 are comparable to super intensive aquaculture conditions. In addition, the glass aquaria without substrate are an extreme environment for the shrimps, leaving them especially vulnerable to cannibalism, thus resulting in a high size distribution and a low survival. Under such super intensive conditions it is necessary to maintain a healthy stock with unsusceptible and resistant animals, possibly promoted by the Friedland clay minerals with and without *Chlorella vulgaris* microalgae.

The applied *Chlorella vulgaris* microalgae originated from a bioreactor attached to a power station fed with CO_2. Algae as a feed additive in aquaculture resulted in positive effects onto the cultivated organisms, such as promoting general health, antibacterial, antifungal and antiviral effects or the supplementation with nutrients, vitamins and other ingredients [39,59-61]. Shrimps are natural algae grazers in shrimp ponds and different algae have been reported to be of importance for the cultivation of larval shrimps [62,63]. The Friedland clay minerals can be used for an efficient and low cost harvesting of microalgae suspension. We could demonstrate that a combination of the *C. vulgaris* microalgae with the beneficial Friedland clay mineral is applicable for shrimp cultivation, additionally making a direct use of bioreactor produced microalgae for a healthy shrimp aquaculture possible. It is recommended that also other microalgae species and their combinations with the Friedland clay minerals should be tested in future studies.

CONCLUSION

White leg shrimp (*Litopenaeus vannamei*) post larvae, 12 days post hatch (PL 1), were fed with different diets including Friedland montmorillonite– illlite/muscovite mixed layer clay minerals as a feed additive with or without the addition of *Chlorella vulgaris* green algae. Positive effects on shrimp growing parameters were found in three independent experiments, especially with an application of 2% clay minerals. The addition of 1% clay minerals in the diets had no positive effects or reduced the performance compared with the control. Addition of microalgae in the diets (mixture with clay mineral) showed comparable effects onto shrimp growth to the 2% montmorillonite treatment. Friedland montmorillonite clay minerals as a feed ingredient resulted in a higher biomass gain after the run of the experiments. Though having a reduced individual weight gain compared with the control also caused by a reduced energy content of the feed, higher survival rates and a more even size distribution of *L. vannamei* resulted in the best performance of the shrimps with 2% montmorillonite. The more homogenous size heterogeneity of shrimps in combination with *Chlorella vulgaris* and montmorillonite (2%/2%) also indicated a positive effect of the feed onto shrimp cohorts under the experimental super intensive conditions. We affiliate this with a suppressed cannibalism in the aquaria. In conclusion, Friedland montmorillonite–illlite/muscovite mixed layer clay minerals have beneficial effects onto White leg shrimp (*Litopenaeus vannamei*) survival and performance at a concentration of 2% with and without addition of *Chlorella vulgaris* green algae.

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