PhD Dissertation

STUDIES ON THE ECO-FRIENDLY AQUACULTURE FOR SHRIMPS USING NATURALLY-OCCURRING INSECT LARVAE

(自然発生する昆虫幼生を利用した環境配慮型エビ養殖に関する研究)

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CHAPTER I

GENERAL INTRODUCTION AND REVIEW

1.1 Shrimp culture in Thailand

Shrimp aquaculture is very important to the socio-economic development in southeastern Asian countries. Among these countries, Thailand has become one of the main countries for world shrimp aquaculture since 1991 (Rosenberry, 1995). In 2008, the production reached 506,602 metric tons in a total 808,300 metric tons of coastal aquatic animal production (Department of Fisheries, 2012). The increasing demand for shrimp production since 1988 has led to increased numbers of farmers and shrimp aquaculture area in Thailand, mostly in the coastal provinces (Department of Fisheries, 2012). Most of the 21,000 shrimp farms with a total area of 48,000 ha are situated in the central region of the country, including 1,222 farms in Songkhla province. During 2007 - 2012, more than 90% of marine shrimps were produced by aquaculture (Department of Fisheries, 2012; 2015; 2016).

Four types of shrimp aquaculture system, depending on the cultured area and stocking density, are used in Thailand, namely 1) extensive (traditional), 2) semi-intensive, 3) intensive and 4) super-intensive (Tookwinas, 2019). Since 1977, most shrimp farming in Thailand has used intensive systems with a pond size of 0.64 – 0.96 ha. Shrimp larvae of black tiger shrimp (*Penaeus monodon*) are stocked at a density of 20-100 postlarvae/m^2. Super-intensive farms have similar pond size, but the stocking density is 80- 200 postlarvae/m^2 for white shrimp (*Litopenaeus vannamei*) culture. This requires very high financial and technical inputs.

Since 2009, the number of semi-intensive farms has remained fairly constant at about 3,000, whilst intensive and super-intensive farms have become dominant, in spite of the decline in number and area since 2012.

In the early stage, the main shrimp species cultured and exported was the black tiger shrimp (*Penaeus monodon*). After 2003, however, because of problems with black tiger shrimp farming, most farmers switched to raise the white shrimp (*Litopenaeus vannamei*). Problems with the outbreak of EMS (Early Mortality Syndrome), however, caused shrimp production to decrease from 2013 until 2015

1.2 Black tiger shrimp (*Penaeus monodon*); biology, aquaculture

Black tiger shrimp (*P. monodon*) is native to the Indo-West Pacific Ocean and Asia including Thailand, Bangladesh, Cambodia, India, Indonesia, Korea, Malaysia, Myanmar, Philipines, Sri Lanka, Taiwan, Vietnam (Dore and Frimodt, 1987; Perez Farfante and Kensley, 1997). This species lives in brackish and marine habitats, including inland saline areas, estuaries, lagoons, coastal areas, mangroves, mud flats, intertidal zone, salt marshes, and inshore marine or benthic zone areas (CABI, 2019). The temperature preferred by this species is 26 – 33 ^{o}C for the adult stage and 28 ^{o}C for broodstock, eggs and larvae (CABI, 2019), with optimal salinity 25 – 35 psu.

Black tiger shrimp was the first species of marine shrimp to be produced commercially in Thailand, where it has been farmed since 1988. The production and value of black tiger shrimp increased year by year until 2000, but then decreased continuously in the 21st century. In 2003, the production and value of black tiger shrimp were much lower than at the peak in 2000 (almost 300,000 tons, 90,000 million Baht). The present farming of black tiger shrimp in Thailand is small and focuses on the production of large-sized shrimps.

1.3 White shrimp (*Litopenaeus vannamei*); biology, aquaculture

White shrimp (*Litopenaeus vannamei*) prefers tropical marine habitats with temperature normally above 20°C throughout the year (FAO, 2019). It becomes mature at the age of 6 – 7 months when males reach 20 g and females 28 g. Larvae of the mysis stage of white shrimps are pelagic but they adapt to benthic in the postlarva stage which is suitable for introduction into aquaculture.

White shrimp has been introduced as an aquaculture species to several areas of the world where it is not native, and is now the most important species in the world, with virtually all production coming from aquaculture (Anderson et al., 2017). World white shrimp production has increased year by year and reached over 4 million tons in 2016 (FAO, 2019). The main producing countries are China, Thailand, Indonesia and Vietnam. Super-intensive white-shrimp culture in Thailand is usually operated in earthen ponds lined with PE (polyethene) plastic and with a high-efficiency aeration system and intensive water quality and shrimp health monitoring. The production and value of cultured white shrimps in Thailand had risen from almost zero to about 600,000 tons and 70,000 million Baht, respectively, in 2011.

1.4 Organic waste in shrimp ponds and its management

The aquaculture of shrimp farms is rapidly developing. Without treatment or management, however, shrimp farming with high stocking density releases into the natural environment much waste water and sludge, which are the main causes of water source pollution (eutrophication) and deterioration (Chua et al., 1992; Phillips et al., 1991; Beveridge et al., 1994; Wu et al., 1994; Hargreaves, 1998; Naylor et al., 1998).

The aquaculture waste has been discharged continuously during shrimp culture. It includes soluble products such as ammonia, urea, carbon dioxide, phosphate and hydrogen sulfide, and particulate wastes, such as shrimp excreta, residual feed, dead plankton, bacterial colonies etc., which often accumulate in the bottom sediment of the pond (sludge), especially in the central part (Nur, 2011).

As reported by several researchers (Boyd, 1992; Satapornvanit, 1993; Funge-Smith and Briggs, 1994, 1998; Lin and Nash, 1996; Paez-Osuma et al., 1997), the intensive degradation of organic matter at the pond bottom and high sediment oxygen demand exceed the oxygen renewal rate. This leads to the development of anoxic conditions in the sediments and at the sediment–water interface, and a large number of potentially toxic materials are generated. Shrimps normally live on or near the bottom of the pond, and are exposed to toxic materials that endanger their well-being, resulting in reduced feeding, slower growth, mortality and possibly higher sensitivity to disease.

Management of waste water and pond waste (sludge), especially of particulate and semi-particulate wastes, is critical in shrimp culture (Behara, 2019). The aim is to minimize coverage of the pond bottom by sludge (Avnimelech and Ritvo, 2003). Resuspension by aerating with airlift pumps or propeller-aspirator aerators (Hopkins et al., 1994) to create a water current gyre, or by raking the pond bottom, is used to prevent sludge accumulation and anaerobic conditions, and allow aerobic decomposition in the culture pond, though this may cause high BOD in the water column and also reduce light penetration. After harvest, the pond waste is usually settled and sun-dried naturally (Behera, 2019), and then dumped outside or into natural water sources. This has an adverse impact on the environmental ecosystem.

When a fraction of the sediment is released with the drained water, especially with the final 20 cm of discharge, into estuaries, rivers or the sea this may severely pollute the environment (Teichert-Coddington et al., 1999). In many farms in Thailand, the sludge from culture ponds is stored in reservoirs, but these are expensive to construct and their storage capacity is limited (Avnimelech and Ritvo, 2003). Alternatively, the sediment can be removed mechanically during culture operation by means of a central drain system or 'shrimp toilet' (Khan, 2018), suction pumps, or rotational pond-waste removal devices.

1.5 Multi-trophic productivity and natural food in shrimp ponds

Shrimp aquaculture ponds generally support a diverse biota (Coman et al., 2003). The daily enrichment of the ponds with large amounts of formulated feed and regular fertilization stimulate the primary productivity and induce the growth of the entire trophic web. Phytoplankton in shrimp ponds is stimulated by the addition of fertilizers and waste products from shrimp (Burford, 1997) and provides food for assemblages of pond zooplankton and epifauna. Zooplankton and epibenthic fauna can contribute to the nutrition of farmed shrimps (Coman et al., 2003).

Gutweed (*Ulva intestinalis*) is a macrophyte green alga (Chlorophyta) that grows abundantly in shrimp ponds and other brackish water areas in Thailand. Green algae can take up various forms of dissolved nitrogen that they can then use as nutrients and fix into forms, especially amino acids and proteins, that can be used as nutrients by animals living in the pond. This leads to the question of whether gutweed can be eaten and used as an alternative source of protein to support growth of cultured shrimps. It is also considered that gutweed can provide shelter for small aquatic fauna such as insect larvae, thereby increasing the abundance of the larvae as natural food for the shrimps. Raking pond

sludge releases nitrogen into the water column and thus could enhance the growth of gutweed.

In Thailand, there are several reports on the productivity in shrimp ponds. The benthic fauna in black tiger shrimp (*Penaeus monodon*) ponds consist of three phyla, and nine group. The dominant benthos are bivalves and chironomids. The benthic organisms significantly increase in relation to an increase in the biomass of gutweed (*Ulva intestinalis*) in the ponds (Suriyaphan et al., 2008). The primary productivity is enhanced by improved water quality (Haque and Rahman, 2008), which in turn enhances secondary and tertiary production of benthic organisms. The management of natural food is of great nutritional importance for the cultured organisms and can contribute to enhancing the economic feasibility of shrimp culture, (Martinez-Cordova et al., 1998a). The contribution varied between 25 and 85% of the shrimp diet, depending on the culture system. The natural food can be stimulated by adding organic and/or inorganic fertilizers, thus increasing the availability of nutrients in the aquatic environment to stimulate primary productivity (Landau 1991).

White shrimps (*Litopenaeus vannamei*) reared in ponds with and without enhancement of natural productivity consumed both natural and artificial foods (Porchas-Cornejo et al., 2012) and showed better growth, confirming the importance of natural foods in shrimp culture ponds; the shrimps tended to consume prey that improved growth performance.

1.6 The potential of insect larvae as feed for shrimp aquaculture

Interest in insect larvae as a protein source has recently increased more and more (Panini et al., 2017a, b; Henry et al., 2018; Iaconisi et al., 2018; Sankian et al., 2018). Some studies have shown the potential of insect larvae as a feed ingredient for fish (Lock

et al., 2016; Belghit et al., 2018; Concetta Elia et al., 2018; Vargas-Abúndez et al., 2019) or shrimp (Panini et al., 2017a, b). The nutritional properties of insect larvae, however, depend on the species of insect (Alegbeleye et al., 2012; Barroso et al., 2014; Sánchez-Muros et al., 2014; Henry et al., 2015). In addition, larvae have already been tested to replace fishmeal for fish farming (Belforti et al., 2015; Roncarati et al., 2015; Sánchez-Muros et al., 2015; Gasco et al., 2016). Finke and Oonincx (2014), as well as other authors (Sharifian Fard et al., 2014), claimed that the nutritional quality of insect proteins has generally been good

Most studies have investigated the incorporation of species of mealworm or black soldier fly larvae in the feed. Larvae of flies such as midges, of the family Chironomidae (Shaw, 1980; Habib et al., 1992; Fernando, 1994; Yusoff et al., 1996; Tdwell et al., 1997) play an important role in the water environment and in aquatic food webs. They represent a major link between primary producers, such as phytoplankton or benthic algae, and secondary consumers (Tokeshi, 1995; Habashy, 2005). These organisms can occupy important positions in the trophic dynamics of aquatic ecosystems, due to their numeric abundance and role in nutrient cycling. The chironomids alter the composition of fine organic matter and supply important subsidies for predators (Silva et al., 2008). Chironomid larvae are an important food source for both tropical and temperate fish (Ciborowski and Corkum, 2003).

Mosquito larvae and pupae live in the aquatic habitat and contribute as a good natural live food for larvivorous as well as carnivorous fish (Blaustein, 1992; Espinoza et al., 1997; Singaravelu et al., 1997) and other aquatic predators (Marian et al., 1983). Further, Degani and Yehuda (1996) reported that mosquito larvae are nutritious due to their high protein content (50%) and that they could be used to formulate artificial diet

for fish, at least to fulfil to some extent the increasing demand for protein (Habib et al., 1992). The larvae and pupae can then be utilized as consumable live food by shrimps, larvivorous and carnivorous fish, aquarium fish and catfish (Shaw and Mark, 1980; Habib et al., 1992; Nuov, 1995; Yusoff et al., 1996; Espinoza et al., 1997). In this way, nutrients from wastes can be recycled to consumable live food for aquatic organisms (Habib et al., 1998).

Live food production in the aquaculture industry is, therefore, surely a very important topic. Larvae of insects such as chironimids (midges) and mosquitoes are important as a natural food source for aquatic animals (Habib et al., 2005; Harbashy, 2005), and are a potential natural food or protein source for shrimp aquaculture.

1.7 Objectives of this study

The objectives of this study are to evaluate the possibility of using insect larvae, in particular chironomid (midge) and mosquito larvae, and gutweed as protein sources for shrimp aquaculture and to elucidate the role of gutweed for recycling nutrients and enhancing the natural populations of these larvae. This study will finally propose an eco-friendly aquaculture for shrimps. The detailed objectives are:

1). To compare the occurrence and levels of different forms of nitrogen in shrimp culture microcosms with different feeding regimes (Chapter II).

2). To evaluate the importance of naturally-occurring insect larvae and gutweed as complementary food for white shrimp (*Litopenaeus vannamei*) aquaculture. (Chapter III, published as Muangyao et al., 2019a).

3). To evaluate these natural foods as protein sources and the possibility for partial replacement of pellet feed (Chapter IV, published as Muangyao et al., 2019b).

4)**.** To investigate the enhancement of the abundance of insect larvae by gutweed and raking of pond sludge (Chapter V, published as Muangyai et al., 2019c).

5). To investigate the role of gutweed in the nitrogen budget of shrimp microcosms with different feeding regimes (Chapter VI).

CHAPTER II

FLUCTUATION OF NITROGEN IN SHRIMP PONDS WITH DIFFERENT FEEDING REGIMES

2.1 Introduction

As mentioned in Chapter 1, the bottom sediment of shrimp ponds has a major influence on shrimp farming. In particular, sludge accumulated in the center of the pond has serious effects on the quality of water in the aquaculture of shrimps (Chanratchakool et al., 1998). Smith (1996) reported that the sediment in black tiger shrimp (*Penaeus monodon*) ponds accumulated 2.25 mg total nitrogen/g, which is significantly higher than that in the mangrove area. The nitrogen is an important factor for the environment quality in shrimp ponds. The sedimentation rates, concentration of total ammonia and total nitrogen in the sediment increased throughout the period of culture and high ammonia concentration in the shrimp-pond water column might be released from the shrimp pond sediment (Tunvilai et al., 1993).

The objective of this chapter is to investigate the fluctuation of nitrogen in shrimp ponds with different feeding strategies. This will generate better understanding of the dynamics of nitrogen accumulation in water and sediment and it will also be useful to improve the feeding practice for shrimp aquaculture described in the following chapters.

2.2 Materials and methods

Preparation of experimental microcosms

The experiments were carried out at the Coastal Aquaculture Research and Development Regional Center 6 (Songkhla), Songkhla Province, in Thailand, by a completely randomized design (CRD) of four feeding regime treatments. The experiment consisted of eight units of brackish water microcosms (surface area 3 m^2, height 80 cm and volume 2,400 L), to which the sludge from a shrimp pond (depth 10 cm) and 300 L of brackish water of 20 psu salinity (depth 10 cm) were added. The sludge was raked once a week for two weeks in order to enhance the process of oxygen transfer and nutrient release. After 3 weeks, the water depth was adjusted to 20 cm by adding a further 300 L of brackish water.

Four sets of microcosms with duplicates for each were prepared. The four treatments were as follows: without pellets (T1, control), with commercial pellet feed (T2), without pellets but with gutweed planted (25 g/m^2) (T3), and with pellets and with gutweed planted (25 g/m^2) (T4). The feeding rates in T2 and T4 were 1kg of pellets per 100,000 shrimps per day (corresponding to the feeding rate in local shrimp farms in Thailand). In the microcosms of T3 and T4, gutweed was planted and allowed to grow for 3 more weeks. Postlarvae of white shrimp *(Litopenaeus vannamei)* (average weight 0.003 g/ind.) were then stocked into each microcosm at a density of 50 individuals/m^2 and cultured for 5 weeks.

Determination of nitrogen in water and sediment

Water in each microcosm was sampled weekly. The water samples were filtered through GF/C filter paper and stored at -20°C before the nitrogen compounds were analysed in the laboratory. The total ammonia nitrogen (TAN) was analysed by a modified indo-phenol blue method (Sasaki and Sawada, 1980), nitrite (NO_2^-) was

analysed by the diazotization method (Bendschneider and Robinson, 1952). Nitrate (NO_3^-) was analysed by cadmium reduction to reduce nitrate to nitrite (APHA, 1985), and NO_2^- was subtracted from the total amount of nitrite produced ($NO_2^- + NO_3^-$). Total dissolved inorganic nitrogen (DIN) was calculated as the sum of TAN, NO_2^- and NO_3^-. Unfiltered water samples were analysed by the persulfate oxidation method (Hansen and Koroleff, 1999) to give the total nitrogen (TN: particulate + dissolved forms). The organic nitrogen (ON) (both particulate and dissolved organic nitrogen) was calculated by the equation: ON = TN – DIN.

Sediment from the central part of each microcosm was sampled weekly. Two replicate samples of each sediment were freeze-dried and ground before the particulate organic carbon (POC) and nitrogen (PON) were analysed by CHN analyser (Truspec CN, LECO).

2.3 Results

Nitrogen in water

The total ammonia nitrogen (TAN) (Fig.2-1A) increased after 1 week of shrimp culture in all treatments, and then decreased in week 2 in the control (T1) and in the gutweed-planted microcosm (T3), though it increased in the microcosms with pellet feeding (T2, T4). The TAN in the microcosm with pellet feeding but without gutweed (T2) was highest in week 4, but stable at around 0.1 to 0.4 mg/l in the other microcosms. At the end of the experiment, TAN was lowest in the control (T1) followed by the gutweed-planted microcosms (T3, T4), and highest in the pellet-feeding microcosm with no gutweed planted (T2) (Fig.2-1A).

Nitrate (Fig. 2-1C) increased in week 1 in all treatments and was highest in the gutweed-planted microcosm (T3). The fluctuation of nitrate was similar to that of nitrite in each treatment, and increased in weeks 4 and 5 in the pellet-feeding microcosms with or without gutweed planted (T2 and T4). The nitrate concentration was quite stable from week 2 until the end of the experiment in the control and gutweed-planted microcosms (T1 and T3). At the end of the experiment, nitrate was highest in the pellet-feeding microcosm (T2) followed by the pellet-feeding microcosm with gutweed planted (T4) (Fig. 2-1C)

The dissolved inorganic nitrogen (DIN) (Fig. 2-2A) increased in all treatments in week 1, and still increased in week 2 in the pellet-feeding microcosm (T2) but decreased in the others.

The organic nitrogen (ON) (Fig. 2-2B) and the total nitrogen (TN) (Fig. 2-2C) were increased in all treatments in week 1. The ON and TN were quite stable from week 2 until the end of the experiment in the pellet-feeding microcosm (T2), but decreased in the other treatments from week 2 to week 3 and then were stable until the end of the experiment. The ON and TN in the pellet-feeding microcosm (T2) were higher than in the other microcosms from week 2 until the end of the experiment.

Nitrogen in sediment

The particulate organic nitrogen (PON) (Fig. 2-3A) in the sediment was about 6 mg-N/g in all treatments at the beginning of the experiment. The PON did not show high fluctuation in the control and gutweed-planted microcosms (T1 and T3). The PON in the sediment in the microcosm with pellet feeding but without gutweed planted (T2) was higher than in the other treatments in weeks 4 and 5 (at the end of the experiment). The C:N ratio (Fig. 2-3B) was about 10:1 at the beginning of the experiment and the ratio did

not show high fluctuation but was a little lower at the end of the experiment than at the beginning.

2.4 Discussion and conclusion

The study in this chapter was carried out to understand the fluctuation of nitrogen in shrimp microcosms with different feeding regimes. There was a high fluctuation of nitrogen compounds in the pellet-feeding microcosm with and without gutweed planted (T2 and T4), especially of harmful forms of ammonia nitrogen (Fig. 2-1A) and nitrite (Fig. 2-1B). Yang et al. (2017) reported that the observed high total ammonia concentration in a shrimp-pond water column might be attributed to the high rate of total ammonia release from the shrimp-pond sediment. The concentration of nitrogen in water, especially organic nitrogen (Fig. 2-2B) or total nitrogen (Fig. 2-1C), was higher in the pellet-feeding microcosm (T2) and lower in the microcosm with pellet-feeding and gutweed planted (T4), similar to that in the microcosms without pellet feeding (T1 and T3).

Sediment in the shrimp microcosm with pellet feeding (T2) had a higher concentration of particulate organic nitrogen (PON) than that in the other feeding strategies (Fig. 2-3A), including the microcosm with pellet feeding and gutweed planted. Organic nitrogen accumulated in sediment at a higher level in the microcosm with pellet feeding (T2). These results indicate that nitrogen from feed was accumulated at a high level in sediment in intensive shrimp ponds. Many studies have been reported about this. Lin and Nash (1996) reported that 26% of the nitrogen applied as feed accumulated in the sediments of intensive shrimp ponds, whereas Funge-Smith and Brigges (1998) reported a value of 24% for shrimp ponds in Thailand. Paez-Osuma et al., (1997) reported that

35.5% of the nitrogen applied as feed accumulated in the sediment in semi-intensive shrimp ponds in Mexico, and Martin et al. (1998) found values up to 38%. Results reported in this chapter, together with information described in these previous reports, strongly suggest that the amount of organic nitrogen in the sediment tended to increase when pellets were given in shrimp pond.

Planting gutweed could reduce the accumulated nitrogen in water and sediment of shrimp ponds and result in a lower nitrogen release into the water column. Seaweeds such as gutweed are macrophytes that can be found in nutrient-enriched shrimp ponds after shrimp harvest. Gutweed, *Ulva intestinalis*, is a widely distributed green alga which grows in some shrimp ponds (Lewmanomont and Ogawa, 1995), and responds to nutrient accumulation by taking up nutrients, growing and storing excess nutrients for future growth. Recently, gutweed is one of the alternative treatments for the rehabilitation of deteriorated shrimp ponds in Thailand. Many studies have revealed benefits of using seaweeds in aquaculture, such as removal of the large amount of ammonia waste excreted, removal of nitrogen and phosphorus in coastal water or reduction of the negative impact of nutrients released from sediment or sludge in the shrimp pond (Neori et al.,1996; Troell et al., 1999; Lombardi et al., 2006). From these previous reports and the present study, it was suggested that planting gutweed in a shrimp pond could minimize the accumulation of nitrogen in the sediment and the water column that occurs with pellet feeding.

In conclusion, results obtained in this chapter clarified the dynamics of nitrogen in sediment in shrimp-rearing ponds with pellet feeding and its influence on the high level of nitrogen in the water column. The strategy of planting gutweed in pellet-feeding shrimp ponds could reduce the accumulation of nitrogen in sediment and also reduce the concentration of nitrogen in the water column. The use of macrophytes such as gutweed

is an alternative practice that is able to balance nutrients in water and sediment in shrimp pond ecosystems.

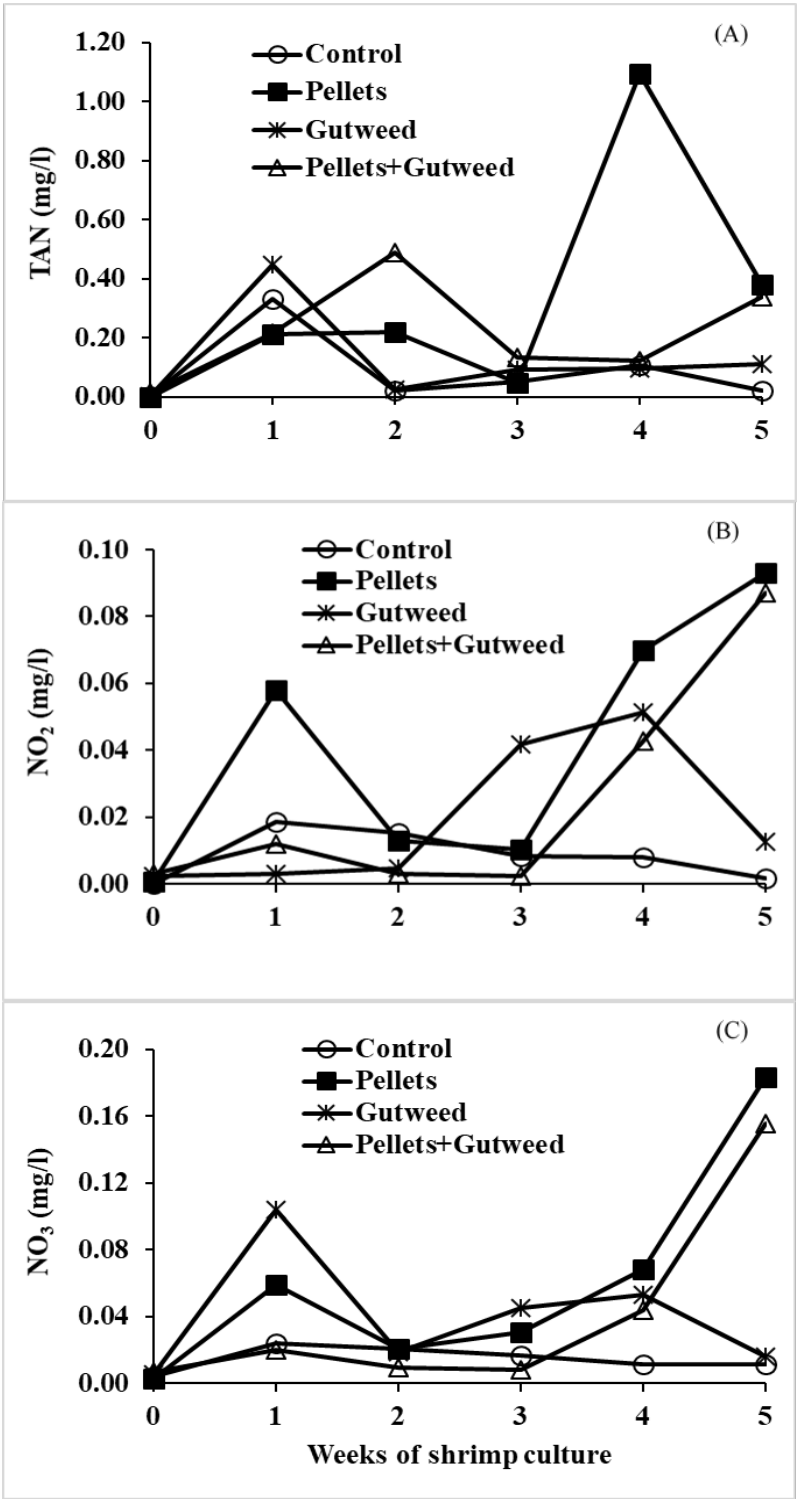

Fig. 2-1 Fluctuation of total ammonia nitrogen (TAN), nitrite (NO_2) and nitrate (NO_3) in water in shrimp microcosms with different feeding regimes [no pellets fed + no gutweed planted (Control); pellets fed (Pellets); gutweed planted (Gutweed); or pellets fed + gutweed planted (Pellets + Gutweed)].

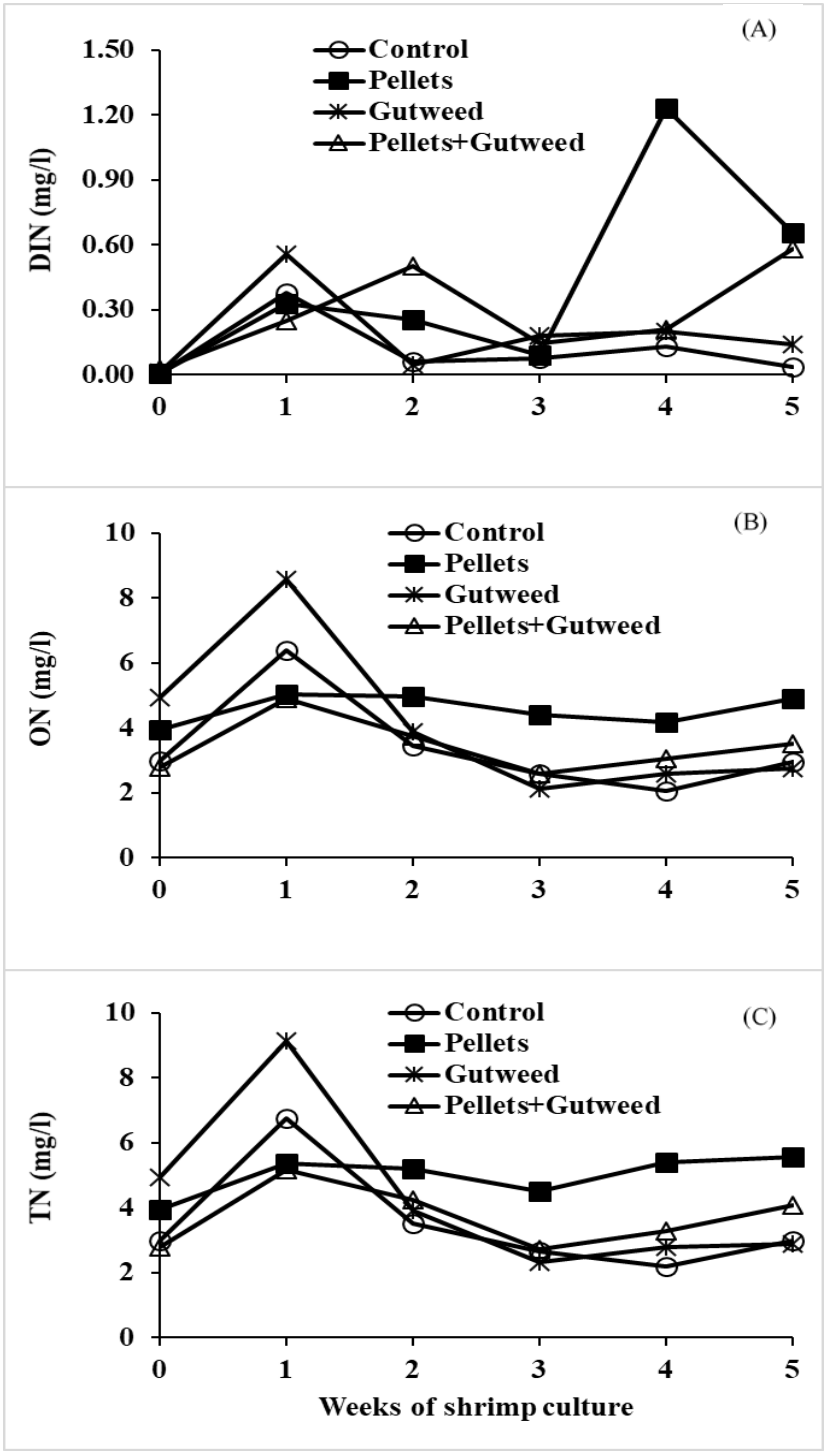

Fig. 2-2 Fluctuation of dissolved inorganic nitrogen (DIN), organic nitrogen (ON) and total nitrogen (TN) in water in shrimp microcosms with different feeding regimes [no pellets fed + no gutweed planted (Control); pellets fed (Pellets); gutweed planted (Gutweed); or pellets fed + gutweed planted (Pellets + Gutweed)].

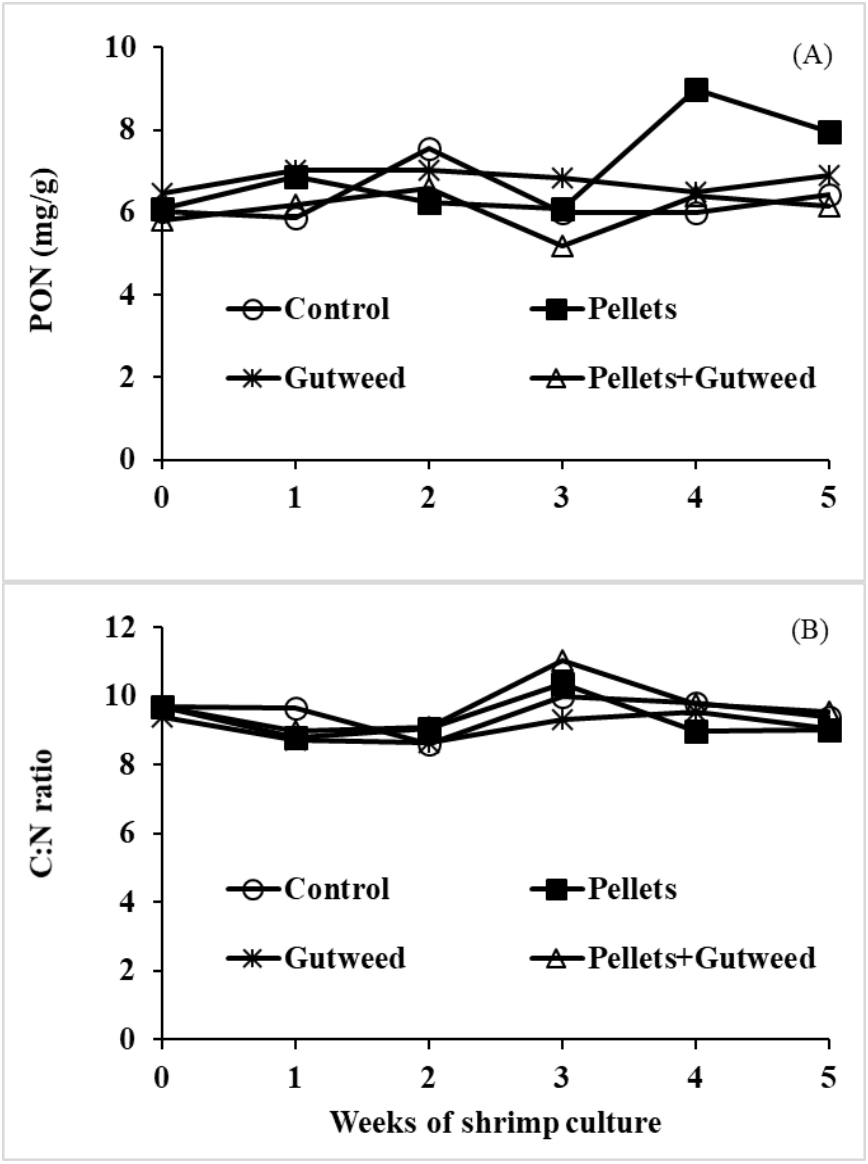

Fig. 2-3 Fluctuation of particulate organic nitrogen (ON) and carbon/nitrogen ratio (C:N) in sediment in shrimp microcosms with different feeding regimes [no pellets fed + no gutweed planted (Control); pellets fed (Pellets); gutweed planted (Gutweed); or pellets fed + gutweed planted (Pellets + Gutweed)].

CHAPTER III

IMPORTANCE OF NATURALLY OCCURRING INSECT LARVAE AND GUTWEED AS COMPLEMENTARY FOOD FOR WHITE SHRIMP (*Litopenaeus vannamei*) AQUACULTURE

3.1 Introduction

White shrimp *Litopenaeus vannamei* is an economically important aquatic resource in many countries and is produced mainly by aquaculture. This has led to increased demand for feed pellets that contain fishmeal as protein source. It is, therefore, of high commercial importance to find alternative protein sources and/or alternative food sources as a substitute or complement for the pellets. This is an urgent requirement to improve feeding practice for shrimp aquaculture (Burford et al., 2004; Hernández et al., 2004; McLean et al., 2006; Nunes et al., 2006; Amaya et al., 2007a, 2007b; Chookird et al., 2010).

One possibility is to use naturally-occurring organisms, such as insect larvae and /or seaweed. There are still few reports on the feeding behavior and the digestion of dominant natural foods by shrimps. More investigations on a laboratory scale are necessary before new complementary food sources can be proposed for white shrimp aquaculture.

The objectives of the study described in this Chapter are (i) to observe feeding behavior and the digestion of chironomid larvae, mosquito larvae, and gutweed by juvenile white shrimps, and (ii) to evaluate the growth and survival rates in each case. Knowledge of these factors will generate better understanding of how to promote shrimp

growth by providing a specific kind of natural food. It will also be useful for improving feeding practice for shrimp aquaculture, and diminishing adverse effects of shrimp culture on the environment.

3.2 Materials and methods

Materials

Larvae of chironomids and mosquitoes used in this study were collected from culture ponds in the finfish hatchery of Coastal Aquaculture Research and Development Regional Center 6 (CARDRC6, Songkhla) in Thailand. Gutweed was collected from an earthen pond in a shrimp farm in Trang province, Thailand. The pellets used were commercially available ones for white shrimp and were obtained from Charoen Pokphand Foods PCL. The chemical composition of the naturally occurring foods and pellets was measured. Analyses of samples were performed at the Chon Buri Aquatic Animal Feed Technology Research and Development Center. Two replicate samples of each naturally occurring food and pellets were freeze-dried and ground before the organic nitrogen content was analysed by CHN analyser (Truspec CN, LECO). Protein contents were calculated by multiplying the amount of organic nitrogen by 6.25. The fat and ash content were measured (AOAC, 2016). The moisture content was also measured by a standard oven-drying method (AOAC, 2005), and dry weight was calculated. Carbohydrate was calculated by the equation

Carbohydrate percentage = 100 - % protein - % fat - % ash.

The chemical compositions are shown in Table 3-1.

Post-larval white shrimps were provided by Blue Gen Solution Hatchery, Songkhla Province, Thailand. The shrimp post-larvae were maintained in the hatchery of the CARDRC6, Songkhla until they were used in the experiments.

Feeding behavior of shrimp

Thirty white shrimps *Litopenaeus vannamei* with initial body weight 0.12 ± 0.03 g and body length 2.7 ± 0.2 cm were starved for 24 h before testing. Each individual shrimp was held in a separate 2-liter container and was fed with 20 pieces, about 0.5 cm length, of one of the natural foods (larvae of chironomid or mosquito, or gutweed). Ten replicates for each were prepared. Numbers of pieces of natural food remaining in each container were counted after 10, 20, 30 min, and then every 30 min until 6 h. The average percentages of food pieces consumed by 30 shrimp replicates were calculated.

Digestion of chironomid larvae, mosquito larvae and gutweed

The time for digestion of each natural food was evaluated by stomach content analysis. Ten shrimps (as ten replicates) for each sampling occasion (7 times) and for each food (3 items) (210 individuals in total) were starved for 24 h before testing. They were fed on a natural food, either one chironomid larva, one mosquito larva or one piece of gutweed (each about 0.5 cm length). After 0.5, 1, 2, 3, 4, 5 and 6 h, shrimps that had eaten the natural food were collected and placed immediately into a salt solution (1 kg salt granules + 3 kg ice cubes + 3 l seawater) at -10^{o}C. The shrimps were then preserved in 10% formalin solution until they could be examined under a compound microscope. Then the foregut was dissected and the contents were washed onto a glass slide. Each food was observed as parts of the body of chironomid larva (head, body and jaw), those of mosquito larva (head, body, anal and siphon tube, and hair) and pieces of gutweed

(Fig. 3-1). The digestion of each food was monitored; for insect larvae, the main body part was observed. The frequency of occurrence was then calculated according to the following equation:

Frequency of occurrence (%) = *Np*/*N'* x 100

Where *Np* = number of stomachs still containing the main part of each food item

N' = total number of shrimp individuals consuming the food

Growth of shrimps

1. Experimental design

The experiment was conducted with a completely randomized design (CRD) of five treatments, with three replicates for each. The five treatments were as follows: shrimps fed with chironomid larvae (T1), fed with mosquito larvae (T2), fed with gutweed (T3), fed with commercial pellets (T4; positive control) or given no food (T5; negative control).

2. Determination of growth

Twenty shrimps, with initial body weight 0.12 ± 0.03 g and body length 2.8 ± 0.2 cm, were cultured in a 35 l glass tank containing water at salinity 20 psu. The water temperature was 28-29^{o}C and about 50% of the water was exchanged every afternoon before the shrimps were fed. Shrimps were fed with the selected food at 08:00, 12:00, 17:00 and 22:00 each day (corresponding to the feeding time in local shrimp farms). The feeding rate was adjusted to provide 46.4 g dietary protein/kg shrimp/day, which corresponds to the recommended protein requirement for maximum growth of juvenile

white shrimp (Kureshy and Davis, 2002). Uneaten food was collected and weighed, and food intake calculated.

Body weights of shrimps were measured every week. Shrimps caught in a hand net were placed on a sheet of paper towel to remove the surrounding water before weighing. Numbers of living shrimps were counted every week for three weeks. After their weights were measured, living shrimps were returned to the glass tank. The average body weight, food conversion efficiency (FCE) and survival rate were calculated at the end of the experiment.

3. Calculations

The following equations were used for calculations.

3.1 Average body weight (g/individual)

= Total wet weight of shrimps / Number of shrimps

3.2 Food conversion efficiency (FCE) (%)

= weight gain (g wet weight) x 100 / food intake (g dry weight)

3.3 Survival rate (%)

= (Final number of shrimps / Initial number of shrimps) × 100

3.4 Statistical analysis

Average body weight, FCE and survival rate in each feeding treatment were analysed statistically by analysis of variance (One-Way ANOVA) and Duncan's New Multiple Range Test (SPSS version 16.0).

3.3 Results

Feeding behavior of shrimps

Changes in the consumption of the three food items are illustrated in Fig. 2 as average per cent for 30 shrimp replicates. When feeding, a shrimp held a larva or a food particle and ate it completely before moving to the next individual item. Of the three food items, chironomid larvae were consumed fastest, followed by mosquito larvae, and gutweed was the slowest. Shrimps consumed chironomid larvae very rapidly; 26.5% of chironomid larvae were eaten within 10 min and more than 50% were eaten within 1 h, whilst only 11.5% of mosquito larvae and 4.0% of gutweed pieces were eaten after 10 min. Thirty percent of shrimps within 3.5 h, and 90% of shrimps at the end of the observation (6 h), had eaten all the 20 chironomid larvae given (Fig. 3-2). None of the shrimps had eaten all 20 mosquito larvae or pieces of gutweed given. Shrimps had eaten 50% of the mosquito larvae within 3 h, whereas 50% of gutweed still remained after 6 h (Fig. 3-2). On average, 98.5% of chironomid larvae were consumed per shrimp, compared with 55% of mosquito larvae and 38% of gutweed (Fig. 3-2).

Digestion of natural foods

When chironomid larvae, mosquito larvae or gutweed were eaten, parts of them remained in the stomach of every shrimp. The percent frequency of occurrence decreased after 0.5 h (Fig. 3-3). The main body part made up 95% of the chironomid larva and 70% of the mosquito larva, by length (see Fig. 1, B2, C3). The main body part of the chironomid larva was consumed and digested very quickly, so that only 20% of the shrimps had any of the main body of the larva left 0.5 h after eating, and the chironomid larva was completely digested within 1 h. In contrast, the main body part of the mosquito larva and the gutweed pieces were more resistant to digestion, but they were digested

completely after 3 h (Fig. 3-3). At the end of the observation period, however, 57.1% of the shrimp stomachs still retained the hair of mosquito larvae (Fig. 3-1, C4), whilst none had any part of chironomid larva or gutweed.

Growth, food conversion efficiency and survival rate of shrimps

Increase in body weight of shrimps fed with one of the three food items is illustrated in Fig. 3-4. Average body weight of shrimps fed with chironomid larvae was significantly higher ($P<0.05$) than those of shrimps fed with mosquito larvae or gutweed and was comparable to that of shrimps that were fed with pellets. The average body weights of shrimps fed with mosquito larvae or gutweed were similarly but significantly higher ($P<0.05$) than those of shrimps given no food, although they were lower than those fed with chironomid larvae or pellet feed (Fig. 3-4). The FCE of shrimps fed on chironomid larvae was highest (147 ± 14.0%) followed by FCE of shrimps fed on pellets (92.5 ± 5.02%), mosquito larvae (73.1 ± 8.44%) and gutweed (44.0 ± 4.38%) (Fig. 3-5). The FCE of shrimps was significantly different between treatments ($P< 0.05$).

Survival rates of shrimps with each of the foods were more than 90% (Fig. 3-5), and were significantly higher than the survival rate of shrimps given no food (60.0 ± 22.9%).

3.4 Discussion and conclusion

In culture ponds, shrimps find and prefer natural foods. In ponds with enhanced natural productivity, they consume more natural food, and stomachs of shrimps from the enhanced ponds contained a higher proportion of natural food than of formulated feed. The shrimps had higher growth when reared in ponds with a high density of natural foods (Porchas-Cornejo, et al., 2012). Several studies have shown the potential value of natural

foods in the nutrition of shrimp (Rubright et al., 1981; Moss and Pruder, 1995; Tacon et al., 2002; Martinez-Cordova et al., 2003, 2005; Gamboa-Delgado, 2014). The main natural food materials consumed by shrimps are zooplankton and benthic organisms (Chiu and Chien, 1992; Martinez-Cordova et al., 1998), though the main species of such organisms are different in different countries.

Results obtained in this study confirmed the omnivorous behavior of juvenile white shrimp in consuming both animal and plant matter, in agreement with previous reports (Dall, 1968; Cockcroft and McLachlan, 1986; Muangyao et al., 2011b). In a previous study it was reported that the stomach contents of shrimp reared in different feeding regimes consisted of a variety of organisms and other organic matter (Muangyao et al., 2011b) and the correlation analysis from that work showed that shrimp growth was closely related to the amount of chironomid larvae in the nutrient material in the microcosms. Shrimps, however, prefer animal-derived to plant-derived food.

In the first hour of observation in the present study, shrimps consumed insect larvae more efficiently than gutweed, and chironomid larvae were the most preferred food for white shrimp. Nearly 100% of chironomid larvae were consumed (Fig. 3-2)

The results given in Fig. 3-3 show that shrimps needed only a short time to digest chironomid larvae, thus shrimps fed with chironomid larvae revealed the best growth (Fig. 3-4) and had the highest FCE (Fig. 3-5). Mosquito larvae have a refractory cuticle of chitin, a polymer of *N*-acetyl-β-D-glucosamine (Merzendorfer and Zimoch, 2003) whilst chironomid larvae are worm-like and have soft skin. These results showed that chironomid larvae are a more suitable food.

Although mosquito larvae and gutweed could not promote shrimp growth rates so much as chironomid larvae or pellets did, they also gave survival rates 30% higher than

those of the shrimp given no food (Fig. 3-6). The results clearly show that chironomid larvae were the best among the three natural foods, but mosquito larvae and gutweed were also promising as a live food for white shrimp and could also be used. These results are useful in relation to providing naturally occurring food for white shrimps and show the potential of insect larvae, especially chironomid larvae, as complementary food for white shrimps.

Insects have received attention in the past decade as protein sources for animals. Some studies showed the potential of insect larvae as a feed ingredient for fish such as rainbow trout (*Oncorhynchus mykiss*) (Borgogno et al., 2017; Concetta Elia et al., 2018), European seabass (*Dicentrarchus labrax*) (Magalhães et al., 2017), blackspot seabream (*Pagellus bogaraveo*) (Iaconisi et al., 2017), Jian carp (*Cyprinus carpio var. Jian*) (Li et al., 2017), Atlantic salmon (*Salmo salar*) (Lock et al., 2016; Belghit et al., 2018) and clownfish (*Amphiprion ocellaris*) (Vargas-Abúndez et al., 2019). Three promising insect species for fish-feed purposes are the black soldier fly (BSF) (*Hermetia illucens*), the common housefly (*Musca domestica*) and the yellow mealworm (*Tenebrio molitor*) because the larvae of these species can grow well on organic waste and produce high-quality protein and fat (Čičková et al., 2015; Nguyen et al., 2015). Use of these species as food in the aquaculture industry would reduce the environmental impact.

The studies cited above focused on the use of cultured insect larvae as a feed ingredient for fish. For white shrimp culture, however, the naturally occurring larvae of chironomid and other insects in shrimp ponds were considered and provided a new concept for the aquaculture. Chironomids are benthic invertebrates which consume mainly sediment detritus (Henriques-Oliveira et al., 2003; Galizzi et al., 2012). The density of chironomid larvae depends mainly on the level of detritus available (Galizzi et

al., 2012). The larvae can reach a high density and remove suspended and/or deposited organic matter, transferring it into the benthic webs (Hirabayashi and Wotton, 1999; Malmqvist et al., 2001). Shrimp ponds accumulate organic matter in the bottom sediment (Funge-Smith and Briggs, 1998; Avnimelech and Ritvo, 2003). This is a source of nutrients that will enhance the density of chironomid larvae in the pond, and will also reduce the amount of organic sediment and thus the impact of waste from shrimp culture on the environment.

Chironomid larvae have all the attributes to be a natural food for juvenile shrimps. The naturally occurring larvae of chironomids in a shrimp pond are beneficial for shrimp culture and could reduce the requirement for pellet feed and help to maintain a suitable controlled environment for the shrimp. The reduction of pellet supply could reduce the accumulation of uneaten feed and thus help to maintain a good environment for shrimp culture throughout the day. There are some reports that chironomids can be parasitic or carry infection to other organisms, but this is specific to a few chironomids species and their host organisms such as insects, sponges, molluscs, bryozoans or fish (Sabine, 2019). The species present in the ponds in the present study were not determined, but the most likely examples are species of genus *Chironomus*, and these have no reported risk of parasitic and other pathogenic infection of shrimps. The chironomid larvae are always present in the shrimp ponds and are a natural food eaten by the shrimp. The present study showed no adverse effects on growth and survival rate of the shrimp.

In conclusion, chironomid larvae were easily digestible and were the most acceptable of the natural foods provided. An abundance of chironomid larvae in a shrimp pond, therefore, can support growth of juvenile shrimps. An abundance of mosquito

larvae and gutweed in the shrimp pond can also serve as additional food to help to support shrimp growth and survival rate.

Gutweed *Ulva intestinalis* is a common green alga which is found in the natural waters and in shrimp-culture ponds in Thailand. Muangyao et al. (2011a) described microcosm experiments containing many kinds of natural food sources including gutweed, insect larvae (chironomid and mosquito larvae) and zooplankton (copepods). They concluded that gutweed might play an important role as the shelter and habitat of chironomid larvae, mosquito larvae, and nauplii of copepods, especially harpacticoids.

The results reported here can be of much benefit by improving feeding practice for shrimp culture. To provide a natural pond environment rich in larvae of insects such as chironomids is a promising way forward for shrimp aquaculture in tropical countries. Evaluation of the nutritional value and protein conversion efficiency of insect larvae such as chironimid larvae by white shrimp is needed and will be reported in the following chapter. This information will be of benefit for providing better understanding of shrimp growth and for application in shrimp-culture systems.

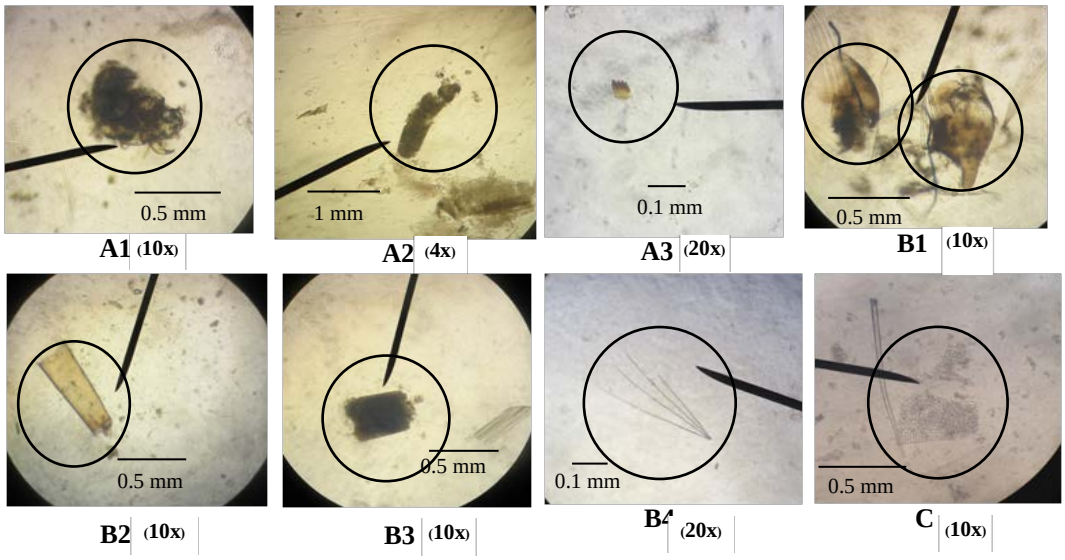

Fig. 3-1 Body parts of chironomid larvae (A1 = head, A2 = body, A3 = jaw); body parts of mosquito larvae (B1 = head, B2 = siphon, B3 = body, B4 = hair); piece of gutweed (C) in the stomach of white shrimps, as observed under the microscope.

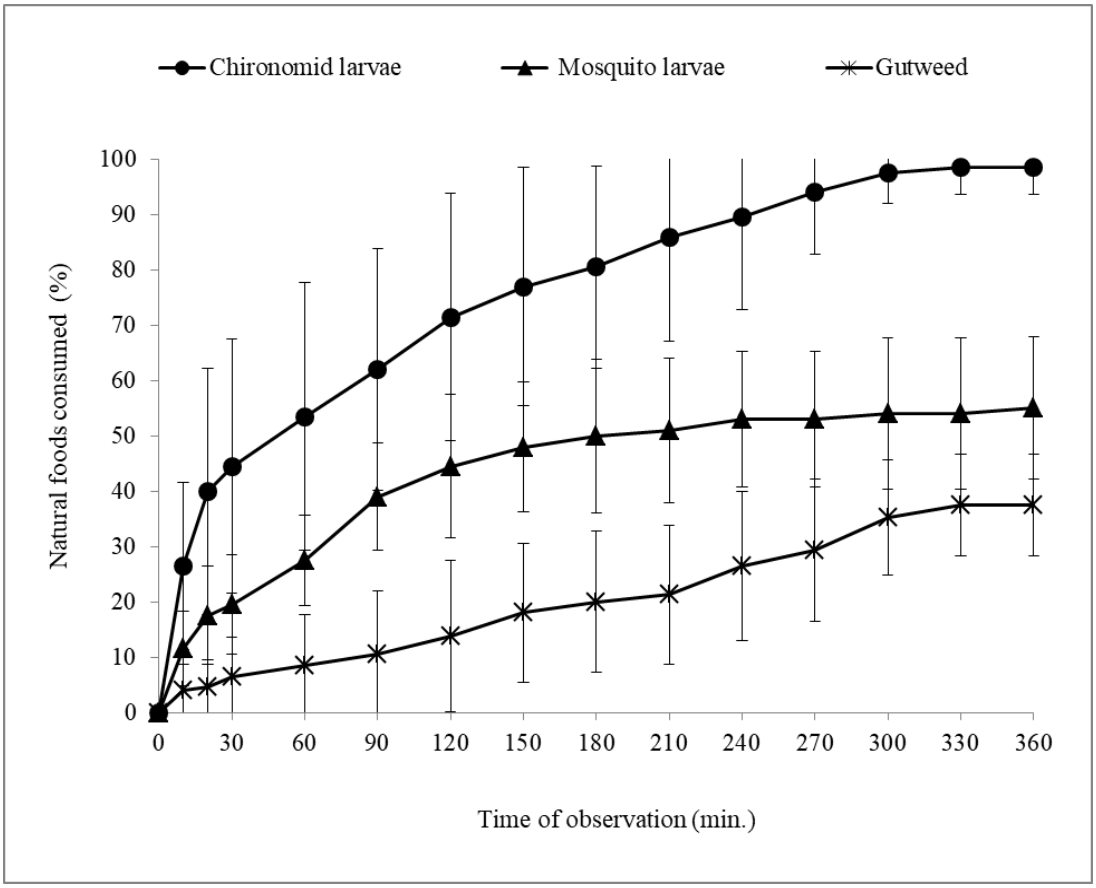

Fig. 3-2 The average percentages of three different natural food items given (chironomid larvae, mosquito larvae and gutweed) consumed by white shrimp, observed at 10, 20, 30 min, and then every 30 min until 6 h.

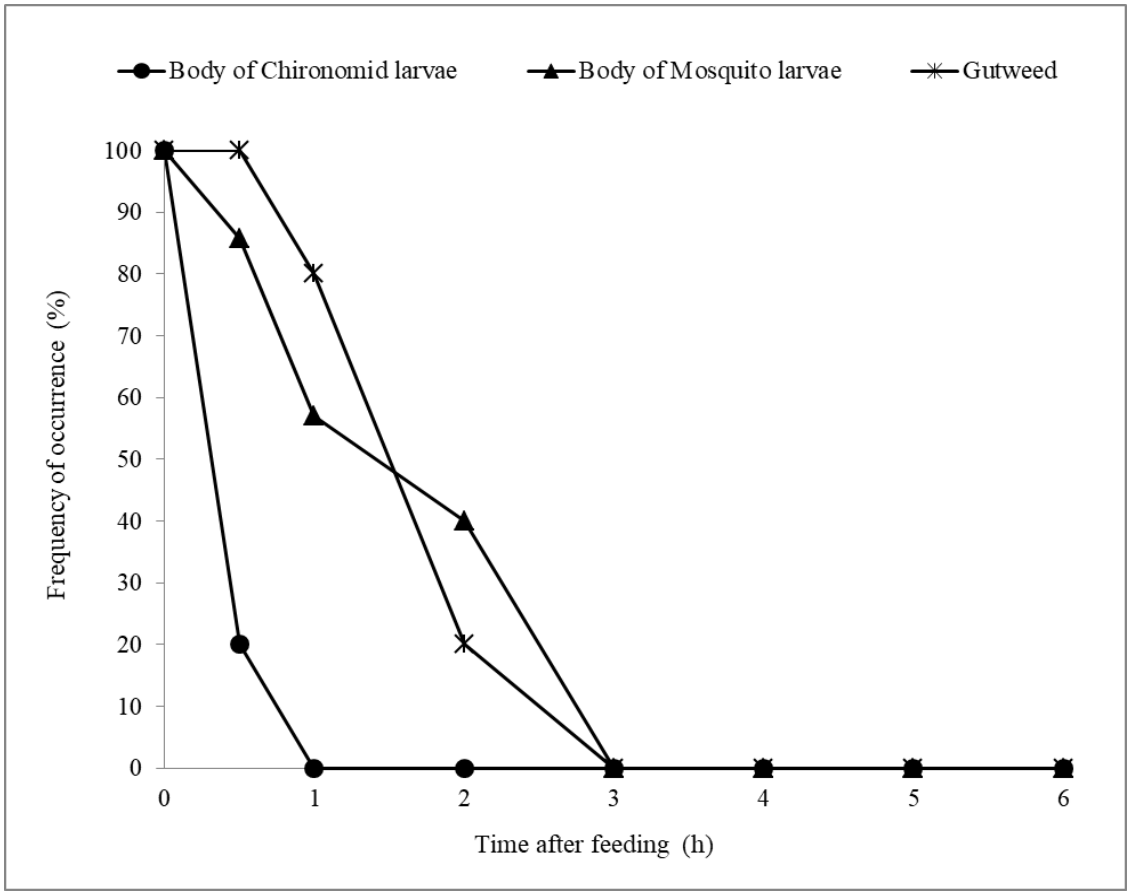

Fig. 3-3 Changes in the percentage of shrimp stomachs that contained the main part of the natural foods chironomid larvae, mosquito larvae or gutweed, observed after 0.5, 1, 2, 3, 4, 5 and 6 h of feeding.

Note: The main part of the body occupies 95% of the whole body in chironomid and 70% of the whole body in mosquito larvae

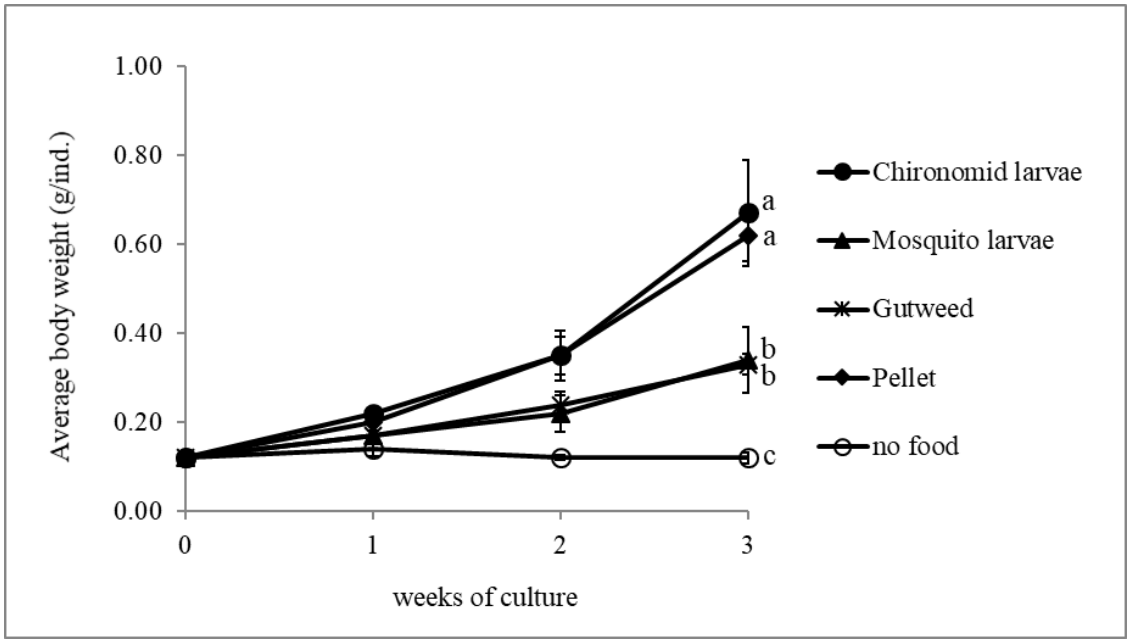

Fig. 3-4 Increase in the average body weight (g/ind.) of white shrimp fed with different foods (chironomid larvae, mosquito larvae, gutweed, pellet or given no food as control)**.**

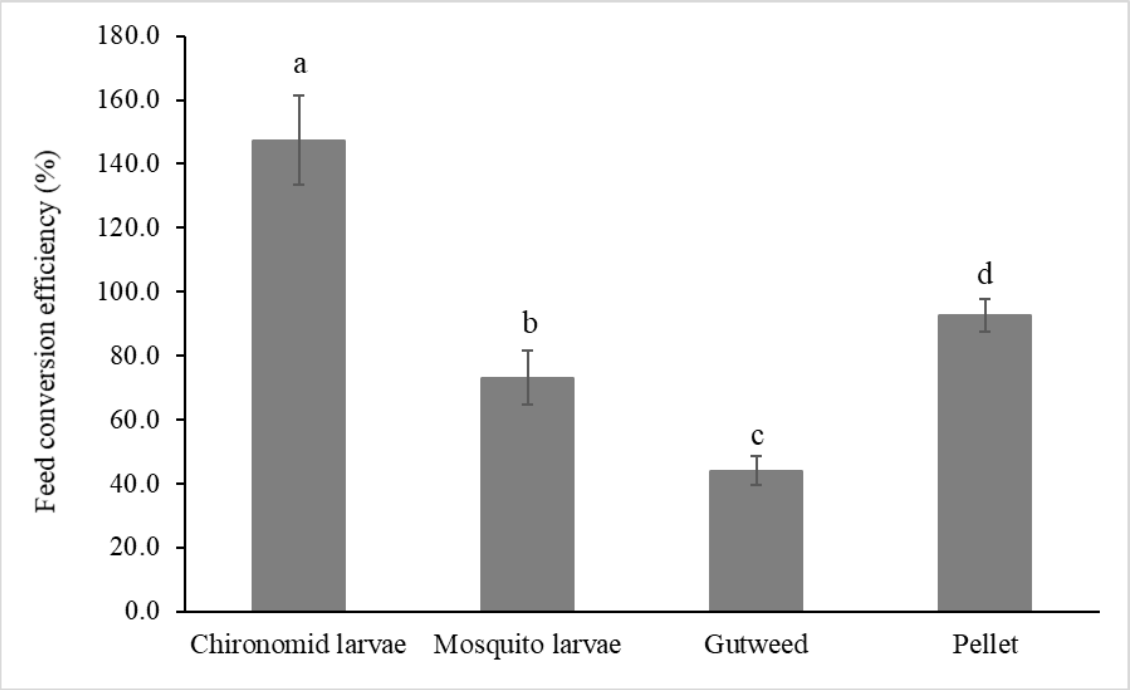

Fig. 3-5 Feed conversion efficiency of white shrimp fed with different foods (chironomid larvae, mosquito larvae, gutweed or pellet).

Different letters above columns indicate significant differences ($P < 0.05$)

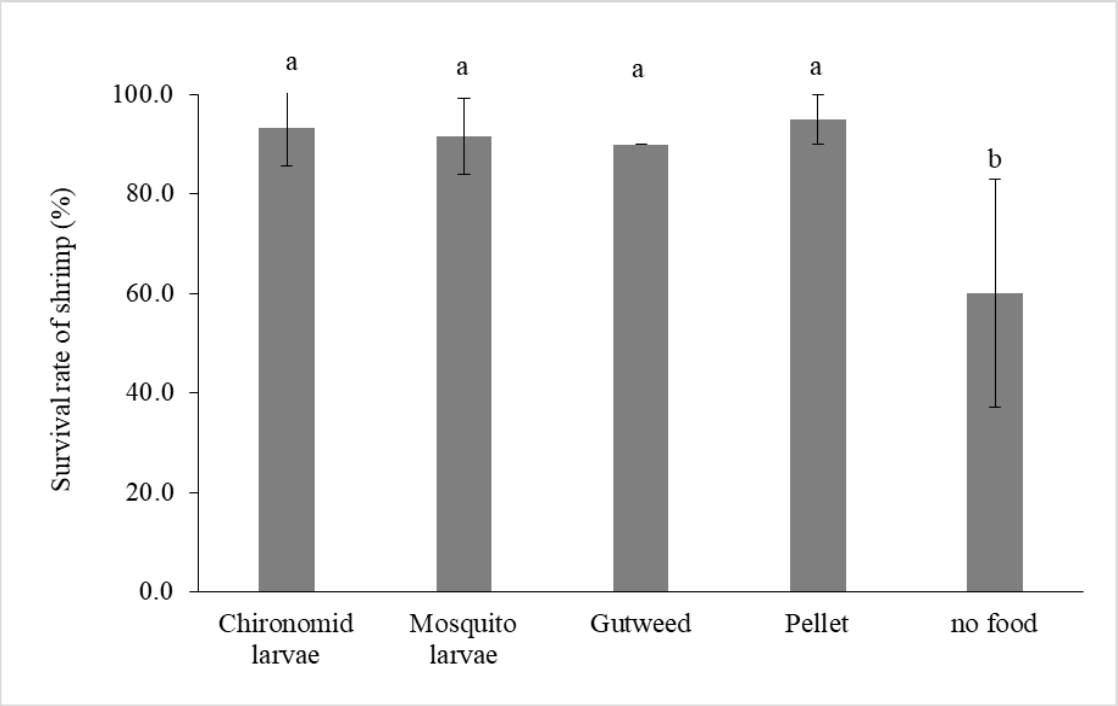

Fig. 3-6 Survival rate of white shrimp fed with different foods (chironomid larvae, mosquitolarvae, gutweed, pellet or given no food as control).

Different letters above columns indicate significant differences ($P < 0.05$)

Table 3-1 Chemical composition of different foods [chironomid larvae (T1), mosquito larvae (T2), gutweed (T3) and pellet (T4)] fed to white shrimps

	Chironomid larvae (T1)	Mosquito larvae (T2)	Gutweed (T3)	Pellet (T4)
Moisture (%)	81.5 ± 0.04	80.5 ± 0.07	94.1 ± 0.02	10.7 ± 0.14
Protein (% dry matter)	62.3 ± 0.18[a]	62.4 ± 0.11[a]	32.7 ± 0.08[b]	37.6 ± 0.48[c]
Fat (% dry matter)	10.4 ± 0.01	14.6 ± 0.16	5.15 ± 0.08	8.11 ± 0.06
Ash (% dry matter)	5.40 ± 0.01	7.22 ± 0.08	20.0 ± 0.04	13.8 ± 0.03
Carbohydrate (% dry matter)	21.9 ± 0.26	15.7 ± 0.27	42.0 ± 0.19	40.5 ± 0.57

CHAPTER IV

EVALUATION OF NATURALLY OCCURRING FOODS IN AQUACULTURE PONDS AS PROTEIN SOURCE AND FOR PARTIAL REPLACEMENT OF PELLETS FOR WHITE SHRIMP *Litopenaeus vannamei*

4.1 Introduction

In the previous Chapter, it was clarified that chironomid larvae are easily digestible and were the most acceptable of the natural foods provided. An abundance of chironomid larvae in a shrimp pond, therefore, can support growth of juvenile shrimps. An abundance of mosquito larvae and gutweed in the shrimp pond can also serve as additional food to help to support shrimp growth and survival rate.

Protein is of greatest nutritional importance and is also responsible for the greatest cost component in any diet (New, 1980). Commercially available pellets are produced with an optimized composition of nutrient sources to give a high protein content as well as amino acid and fatty acid profiles that are suitable for aquaculture. The value of the naturally occurring foods as protein sources should still be assessed more. It is also fundamental to develop alternative food and protein sources and to evaluate the nutritional value of the protein in these sources.

The objectives of the study described in this chapter are to evaluate (i) the protein content and the amino acid composition of chironomid larvae, mosquito larvae, and gutweed, (ii) the effectiveness of the protein for white shrimps fed on these natural foods,

and (iii) the effects of combinations of two different foods, replacing, in part, feed pellets with the natural food.

4.2 Materials and methods

Materials used and the experimental design

Larvae of chironomids and mosquitoes used in this study were collected from culture ponds in the finfish hatchery of Coastal Aquaculture Research and Development Regional Center 6 (CARDRC6, Songkhla) in Thailand. Gutweed samples were collected from a pond in a shrimp farm in Trang Province, Thailand. All these materials were kept in the refrigerator ($4^{o}C$) until they were used for feeding experiments. The pellets used were commercially available ones for white shrimp and were obtained from Charoen Pokphand Foods PCL. Post-larval white shrimps were provided by Blue Gen Solution Hatchery, Songkhla Province, Thailand. The shrimp post-larvae were maintained in the hatchery of the CARDRC6 until they were used in the experiments.

Experiment 1: Evaluation of individual food items

The experiment was conducted with a completely randomized design (CRD) of five treatments, with three replicates for each. The five treatments were as follows: shrimps fed with chironomid larvae (T1), fed with mosquito larvae (T2), fed with gutweed (T3), and, as controls, fed with commercial pellets (T4) or given no food (T5). Preparation of shrimps and feeding experimental design were similar to those described in the previous chapter. In brief, twenty shrimps (initial body weight 0.12 ± 0.03 g and body length 2.8 ± 0.2 cm) were cultured in a 35 l glass tank containing seawater of 20 psu.

Shrimps were fed with the selected food and the feeding rate was adjusted to provide 46.4g dietary protein/kg shrimp/day, which corresponds to the recommended protein requirement for maximum growth of juvenile white shrimp (Kureshy and Davis, 2002). This feeding rate was calculated from the initial weight of shrimps at the beginning of each week and the rate was fixed for the week until the shrimps were weighed again.

Body weights of shrimps were measured every week. Shrimps caught in a hand net were placed on a sheet of paper towel to remove the surrounding water before weighing. The number of shrimps was also counted every week for three weeks. After weighing, living shrimps were returned to the container. Uneaten food was collected and weighed to calculate food intake rate and protein intake. The organic nitrogen content and amino acid composition of the shrimps were determined at the end of the feeding experiments. Average growth rate (AGR), food conversion ratio (FCR), food conversion efficiency (FCE), net protein utilization (NPU) and protein efficiency ratio (PER) were calculated.

Experiment 2: Evaluation of the effects of food combinations

The experiment was conducted with a completely randomized design (CRD) of five treatments, with three replicates for each. The five treatments were as follows: shrimps fed on pellets (T6), fed on 50% of pellets and 50% of chironomid larvae (T7), fed on 25% of pellets and 75% of chironomid larvae (T8), fed on 50% of pellets and 50% of mosquito larvae (T9) and fed on 25% of pellets and 75% of mosquito larvae (T10).

Twenty shrimps, with initial body weight of 0.28 ± 0.00 g, were cultured in a 35 l glass tank containing water at salinity 20 psu. The water temperature was 28-29^{o}C.

About 50% of the water was exchanged every afternoon before the shrimps were fed. Shrimps were fed with the selected food at 08:00, 12:00, 17:00 and 22:00 each day (corresponding to the feeding time in local shrimp farms). The feeding rate was adjusted as described for experiment 1. Pellets and insect larvae (wet matter) were given separately but at the same time. Uneaten food was collected and weighed to calculate protein intake. The body weights of shrimps were measured and numbers of living shrimps were counted every week. The organic nitrogen content and amino acid composition of the shrimps were determined at the end of the feeding experiments. The survival rate, PER and AGR were calculated.

Determination of protein content and amino acid composition of natural foods

Analyses of samples (naturally occurring foods and shrimps) were performed at the Chon Buri Aquatic Animal Feed Technology Research and Development Center. Two replicate samples of each naturally occurring food, pellets and shrimps were freeze-dried and ground before the organic nitrogen content was analysed by CHN analyser (Truspec CN, LECO). Protein contents were calculated by multiplying the amount of organic nitrogen by 6.25. Amino acid compositions were analysed for samples of each naturally occurring food, pellets and shrimps. The samples were hydrolysed with hydrochloric acid to give free amino acids and the compositions were then analysed by high-performance liquid chromatography (HPLC, Agilent 1100 series), with post-column derivatization (In-house method based on Bidlingmeyer et al., 1987). The moisture content was also measured by a standard oven-drying method (AOAC, 2005), and dry weight was calculated.

Net protein utilization (NPU) and protein efficiency ratio (PER) were calculated as indicators of the nutritional value of proteins and used to evaluate the effectiveness of the protein sources.

Calculations

Food intake rate (g/g shrimp/day)

= total food eaten (g wet weight) /total shrimp weight (g wet weight) / number of days

Food conversion ratio (FCR)

= food intake (g wet weight) / weight gain (g wet weight)

Food conversion efficiency (FCE) (%)

= weight gain (g wet weight) x 100 / food intake (g dry weight)

Protein intake (g/g shrimp/day)

= total protein eaten (g dry weight) / total shrimp weight (g wet weight) / number of days

Average growth rate (AGR) (% body weight gain/day)

= total weight gain (g wet weight) / initial weight (g wet weight) / days x 100

Survival rate (%)

= (final number of shrimps / initial number of shrimps) × 100

Net protein utilization (NPU) (%)

= total protein gain in the shrimps (g dry weight) / protein intake (g dry weight) × 100

Protein efficiency ratio (PER)

= weight gain (g wet weight) / protein intake (g dry weight)

Statistical analysis

Food intake rate, protein intake, FCR, FCE, AGR, NPU, PER and protein content of shrimps in each feeding treatment were analysed statistically by analysis of variance (One-Way ANOVA) and Duncan's New Multiple Range Test (SPSS version 16.0).

4.3 Results

Protein content and amino acid composition

Protein content of the three food items and pellets is shown in Table 4-1 as percentage of dry and wet weight. Chironomid and mosquito larvae had high protein content of ca. 62% dry matter and these values were higher than the protein content of pellets on a dry weight basis, but lower when based on wet weight because of the higher water content of the live larvae. The protein content of gutweed was lower than that of insect larvae and pellets, on both dry and wet matter basis.

The content and amino acid composition of each food are shown in Table 4-1. The content of essential amino acids (EAA) and non-essential amino acids (NEAA), and ratio of the essential to non-essential amino acids (EAA/NEAA) in larvae of both chironomid and mosquito were 30-31%, 34% and 0.9, respectively, and were approximately similar. These values were lower than in pellets but higher than in gutweed

(Table 4-1). The sulphur amino acids methionine (essential) and cysteine (non-essential) and aspartic acid were higher in the larvae than in pellets.

The protein content of shrimps cultured with these foods is shown in Table 4-2. Shrimps fed on chironomid larvae had significantly higher protein content ($P<0.05$) than those fed on mosquito larvae or pellets. Shrimps fed on gutweed or no food, however, had significantly lower protein content ($P<0.05$) than those fed on any other food (Table 4-2).

The concentration of EAA in shrimps fed on mosquito larvae or pellets was similar, and higher than in shrimps fed on chironomid larvae or given no food but lower than in shrimps fed on gutweed. The EAA/NEAA ratio of shrimps fed on chironomid larvae, gutweed and shrimps given no food was higher than for shrimps fed on mosquito larvae or pellets (Table 4-2).

Growth and protein nutrition of shrimps

Food intake rate, protein intake, FCR, FCE, AGR, NPU and PER of shrimps fed on different foods are shown in Table 4-3. Food intake rates of shrimps differed significantly among treatments T1, T2 and T3. Shrimps showed the highest food intake rate from gutweed and the lowest from pellets. Rates from shrimps fed on larvae of chironomid and mosquito were between these two values and were approximately equal (Table 4-3). Shrimps could not eat all of the gutweed they were fed, even though they ate all the time and food was always present in their gut. The protein intake of shrimps fed on mosquito larvae was similar to that of shrimps fed on chironomid larvae, but higher than for shrimps fed on pellets ($P<0.05$). The protein intake of shrimps fed on gutweed was lowest ($P<0.05$) (Table 4-3). The FCR of shrimps fed on pellets was lowest (1.22 ±

0.07). The FCR values for chironomid larvae (3.70 ± 0.36) were lower (P<0.05) than those for mosquito larvae (7.09 ± 0.77) and gutweed (38.6 ± 4.00), and higher than those for pellets. Statistical analysis, however, showed that the difference between feeding chironomid larvae and pellets was not significant (P>0.05) (Table 4-3). The FCE of shrimps fed on chironomid larvae was highest (147± 14.0%) followed by FCE of shrimps fed on pellets (92.5 ± 5.02%), mosquito larvae (73.1 ± 8.44%) and gutweed (44.0 ± 4.38%) (Table 4-3). The FCE of shrimps was significantly different between treatments (P<0.05) (Table 4-3). The AGR of shrimps fed on chironomid larvae was significantly higher (P<0.05) than for those fed mosquito larvae or gutweed, and comparable to AGR for shrimp fed on pellets (Table 4-3). Nevertheless, AGRs of shrimps fed on mosquito larvae or gutweed were similar and significantly higher (P<0.05) than those of shrimps given no food (Table 4-3). The NPU and PER of shrimps fed on chironomid larvae or pellets were significantly (P<0.05) higher than all the others. (Table 4-3).

Effects of food combinations

Protein intake, PER, AGR, and survival rates of shrimps fed on different food combinations are shown in Table 4-4. The highest protein intake was seen for shrimp fed on 100% mosquito larvae, but there was no significant difference among all others (P>0.05). The PER of shrimps fed on 100% pellets was 2.46. Feeding a combination of 50% pellets and 50% chironomid larvae gave the highest PER, the AGR being significantly higher (P<0.05) than for shrimps fed on 100% pellets. Other feeding combinations, namely 25% pellets and 75% chironomid larvae or mosquito larvae gave similar (P>0.05) PER and AGR to feeding pellets alone (Table 4-4). Survival rates were not significantly different among all the treatments (P>0.05) (Table 4-4).

4.4 Discussion and conclusion

The potential of naturally-occurring materials as additional food for shrimp aquaculture has been studied (Tacon et al., 2002; Martinez-Cordova et al., 1998, 2003, 2005; Gamboa-Delgado, 2014). Insect larvae such as chironimid and mosquito larvae are important as a natural source of food for aquatic animals (Habib et al., 2005; Harbashy, 2005). Interest in the importance of insect larvae as a protein source has recently increased more and more (Panini et al. 2017a,b; Henry et al. 2018; Iaconisi et al. 2018; Sankian et al. 2018). Some studies have shown the potential of insect larvae as a feed ingredient for fish (Lock et al., 2016; Belghit et al., 2018; Concetta Elia et al., 2018; Vargas-Abúndez et al., 2019) or shrimp (Panini et al. 2017a,b). The nutritional properties of insect larvae, however, depend on the species of insect (Alegbeleye et al., 2012; Barroso et al., 2014; Sánchez-Muros et al., 2014; Henry et al., 2015).

Chironomid larvae have a high content of protein and essential amino acids, and of lipid, including essential fatty acids (Habib et al., 1997; Habashy, 2005), vitamins, and minerals (McLamey et al., 1974; Habib et al., 1997). Habib et al. (1997) stated that, in aquaculture practice, minerals might be supplemented to fish and shrimps by feeding chironomid larvae. Shrimps feeding on insect larvae, therefore, should not be deficient in any other nutrients.

Martinez-Cordova et al. (1998) reported that supplementation with natural food seemed to be a better feeding strategy for white shrimp farmed in culture ponds and gave the lowest (i.e. best) FCR (food conversion ratio). It was shown in Chapter III that naturally-occurring larvae of chironomids and mosquitoes, and the green alga gutweed (*Ulva intestinalis*) were easily digested by white shrimp; chironomid larvae, in particular,

were the most preferred by shrimps. In that study, shrimps had eaten 98.5% of chironomid larvae within 6 hr, but only 58% of mosquito larvae and 38% of gutweed, respectively.

Chironomid larvae have a high protein content of 62% (Table 1), similar to that of fishmeal (60-72%) (Cho and Kim, 2011), which is the main protein source in shrimp aquaculture pellets (Suárez et al., 2009, Olmos et al., 2011). This indicated that chironomid larvae could be used as protein source. Compared to pellets, they have a higher protein content (dry matter basis), but the quality of protein from pellets is better. The concentrations of EAA and NEAA and the ratio of EAA/NEAA in the chironomid larvae and mosquito larvae were lower than in the pellets, but the quality of protein in pellets depends on the quality of the protein sources used (Tantikitti et al., 2016). The concentrations of EAA and NEAA in the chironomid and mosquito larvae were higher than those reported in another study (Tantikitti et al., 2016) for pellets which were formulated with premium-grade fishmeal. The EAA/NEAA ratios of those larvae were also higher than those for five diets containing fishmeal from different sources (Tantikitti et al., 2016).

The protein content and the concentrations of EAA and NEAA in the chironomid and mosquito larvae were higher than in gutweed (Table 1). Plant products usually have lower content of protein and amino acids than animal products (Hardy, 2010; Tantikitti, 2014)

The protein content of insects and their larvae depends on the species of insect. The adult variegated grasshopper *(Zonocerus variegatus*) had 61.5% crude protein (Alegbeleye et al., 2012). Other insect products such as black soldier fly larvae (*Hermetia illucens*) (42.1%), housefly maggot meal (*Musca domestica*) (50.4%), adult of house cricket (*Acheta domesticus*) (63.3%), and silkworm pupae meal (*Bombyx mori*

(Lepidoptera) (60.7%) have different levels of crude protein (Tran et al., 2015). Results obtained in the present study showed that the protein content of larvae of chironomid and mosquito were higher than in most of these insects and larvae, almost reaching the level reported for the adult house cricket.

The NPU is directly related to shrimp growth. Shrimps were able to convert protein from the chironomid larva into that in the shrimp body at a higher level than from the other natural foods and at a similar level to that of protein from pellets (Table 4-3). Even though the NPU of shrimps fed on chironomid larvae was not significantly different to that of shrimps fed on pellets, the FCE in shrimps fed on chironomid larvae was higher. Pellets are produced to be a rich source of protein, and to contain other nutrient sources suitable for shrimp growth. The larvae also have high contents of essential amino acids, lipid, and essential fatty acids (Habib et al., 1997; Habashy, 2005), vitamins and minerals (McLamey et al., 1974; Habib et al., 1997). The nutritional quality of the proteins in the pellets used in the present study is better than that of the protein in the chironimid larvae but, nevertheless, chironomid larvae are promising as a source of food to stimulate shrimp growth.

Mosquito larvae also have a high protein content (Table 4-1) and an amino acid profile like that of chironomid larvae (Table 4-2), although shrimps fed with mosquito larvae revealed lower growth and lower NPU. The FCE of mosquito larvae was lower than that of chironomid larvae, but it was reported in Chapter III that shrimps need a longer time to digest mosquito larvae than chironomid larvae, which are a more acceptable food. Shrimps fed on chironomid larvae had high growth, and their growth was comparable to that of shrimps fed on pellets.

Gutweed had the lowest protein content and concentration of EAA and NEAA (Tables 4-1 and 4-2), so shrimp have to eat more gutweed to obtain a high enough level of protein. Gutweed showed the highest food conversion ratio (FCR) and the lowest FCE (Table 4-3). In this case shrimps could not eat all of the gutweed fed, so the growth of shrimps fed gutweed was lowest.

Evaluation of the effect of feeding combinations of foods revealed that shrimps fed the combination of chironomid larvae and pellets (50% or 75% of insect larvae + 50% or 25% of pellets) had higher AGR than shrimps fed on 100% pellets (Table 4-4). Feeding on 50% of chironomid larvae and 50% of pellets was particularly effective, even more so than feeding pellets alone (Table 4-4). These results show that feeding a combination of pellets and chironomid larvae can be more effective than feeding pellets or chironomid larvae alone; experiment 1 showed that pellets and chironomid larvae are similarly effective. The shrimps appeared to incorporate more protein from the combination and to have enhanced EAA content. Feeding mosquito larvae was also effective, but the shrimps prefer to eat chironomids. Pellets have higher concentrations of EAA and NEAA and higher ratios of EAA/NEAA but chironomid larvae have higher concentrations of methionine, aspartic acid and cysteine. So, the combination of pellets and chironomid larvae could provide a more suitable profile of amino acids than pellet or larvae alone. The combinations of pellets with up to 75% of chironomid or mosquito larvae gave similar growth to shrimps fed on pellets alone (Table 4). This indicated that up to 75% of the pellet feed could be replaced by these insect larvae, thus reducing the cost of providing feed for shrimp aquaculture.

The result obtained here does raise some questions, for example whether there may be some as yet unidentified factor in the chironomid larvae that stimulates the uptake

of protein from pellets, or whether feeding the combination of pellets and insect larvae may give a more suitable nutritional balance for juvenile white shrimp. In any case, up to 75% of pellet feed could be replaced by the insect larvae without any negative effect on shrimp growth. The observation reported in this study may, therefore, be important for future improvements.

It can be concluded that stimulating the occurrence of chironomid larvae in a shrimp pond will support more shrimp growth than simply feeding pellets alone. The way to stimulate the occurrence of these insect larvae in ponds should be investigated. Knowledge of this would be of benefit by improving pond preparation practice in a way that would support shrimp growth and reduce the need for pellets, and thus reduce the production cost of shrimp culture.

Table 4-1 Protein content, essential amino acid and non-essential amino acid composition of different foods [chironomid larvae (T1), mosquito larvae (T2), gutweed (T3) and pellets (T4)] fed to white shrimps

	Chironomid larvae (T1)	Mosquito larvae (T2)	Gutweed (T3)	Pellet (T4)
Protein content				
% dry matter	62.3 ± 0.18[a]	62.4 ± 0.11[a]	32.7 ± 0.08[b]	37.6 ± 0.48[c]
% wet matter	11.5 ± 0.03[a]	12.1 ± 0.02[a]	1.94 ± 0.00[b]	33.5 ± 0.43[c]
Essential amino acid: EAA (g/100 g protein)				
Arginine	3.61	4.14	2.83	7.03
Histidine	1.82	2.11	0.73	2.20
Threonine	3.06	3.16	2.17	3.33
Phenylalanine	3.73	3.20	2.18	4.03
Valine	3.07	3.19	2.08	3.62
Methionine	2.99	3.62	0.90	0.70
Isoleucine	2.96	2.61	1.44	3.38
Leucine	4.41	4.42	2.82	6.10
Lysine	4.54	4.51	1.77	8.37
Total EAA	30.19	30.96	16.91	38.75
Non-essential amino acid: NEAA (g/100 g protein)				
Aspartic acid	7.31	5.89	5.62	0.00
Glutamic acid	8.85	9.48	4.74	14.99
Serine	3.02	2.96	2.10	3.45
Glycine	2.85	2.75	2.20	5.83
Alanine	5.71	4.81	3.16	6.80
Proline	2.57	2.96	1.41	4.73
Tyrosine	2.67	3.61	1.29	4.28
Cysteine	0.67	0.69	0.55	0.10
Taurine	ND	0.83	ND	ND
Total NEAA	33.64	33.98	21.07	40.18
EAA/NEAA	0.90	0.91	0.80	0.96

Note: Numbers in the same row with different superscripts are significantly different ($P<0.05$), (N = 2 for protein and moisture, N = 1 for amino acid), ND = Not detected

Table 4-2 Protein content, essential amino acid and non-essential amino acid composition of white shrimps after feeding for 3 weeks with different foods [chironomid larvae (T1), mosquito larvae (T2), gutweed (T3) or pellet (T4) or given no food as control (T5)]

	Shrimp fed on chironomid larvae (T1)	Shrimp fed on mosquito larvae (T2)	Shrimp fed on gutweed (T3)	Shrimp fed on pellet (T4)	Shrimp with no food (T5)
Protein content					
% dry matter	71.4 ± 0.30[a]	70.0 ± 0.00[b]	68.8 ± 0.12[c]	70.0 ± 0.25[b]	58.9 ± 0.00[d]
% wet matter	15.46 ± 0.06[a]	15.82 ± 0.00[b]	15.11 ± 0.03[c]	15.70 ± 0.06[d]	9.09 ± 0.00[e]
Essential amino acid: EAA (g/100 g protein)					
Arginine	6.49	4.34	6.13	3.15	3.78
Histidine	1.78	1.97	1.89	2.13	1.80
Threonine	3.15	4.87	3.46	3.68	3.34
Phenylalanine	3.46	3.96	3.78	4.14	3.59
Valine	3.21	3.81	3.58	3.84	3.31
Methionine	4.72	2.22	5.40	2.16	2.06
Isoleucine	3.10	4.34	3.22	5.77	5.56
Leucine	5.40	7.97	5.81	7.10	5.67
Lysine	5.47	5.21	5.80	6.53	6.98
Total EAA	36.77	38.7	39.08	38.49	36.1
Non-essential amino acid: NEAA (g/100 g protein)					
Aspartic acid	7.28	9.21	8.30	9.22	7.25
Glutamic acid	11.89	13.36	12.79	14.16	10.42
Serine	3.28	3.83	3.50	3.86	3.44
Glycine	6.49	7.57	6.77	7.01	6.10
Alanine	5.34	6.50	5.28	6.33	4.30
Proline	2.97	6.99	3.17	3.85	2.83
Tyrosine	2.90	3.49	2.93	3.47	3.01
Cysteine	0.82	0.97	0.89	0.90	1.08
Taurine	1.22	1.43	1.30	1.08	2.16
Total NEAA	42.19	53.34	44.93	49.87	40.59
EAA/NEAA	0.87	0.73	0.87	0.77	0.89

Note: Numbers in the same row with different superscripts are significantly different ($P<0.05$), (N = 2 for protein and moisture, N = 1 for amino acid), ND = Not detected

Table 4-3 Comparison of food intake rate, protein intake, food conversion ratio (FCR), food conversion efficiency (FCE), average growth rate (AGR), net protein utilization (NPU) and protein efficiency ratio (PER) of white shrimp fed for 3 weeks with chironomid larvae (T1), mosquito larvae (T2), gutweed (T3), pellet (T4) or given no food (T5) as control

	Chironomid larvae (T1)	Mosquito larvae (T2)	Gutweed (T3)	Pellet (T4)	No food (T5)
Food intake rate (g wet weight/ g shrimp/day)	0.38 ± 0.00^{a}	0.38 ± 0.01^{a}	1.83 ± 0.02^{b}	0.12 ± 0.00^{c}	-
Protein intake (g wet weight/g shrimp/day)	0.044 ± 0.000^{ab}	0.046 ± 0.000^{b}	0.035 ± 0.000^{c}	0.042 ± 0.001^{a}	-
FCR	3.70 ± 0.36^{ab}	7.09 ± 0.77^{b}	38.6 ± 4.00^{c}	1.22 ± 0.07^{a}	-
FCE (%)	147 ± 14.0^{a}	73.1 ± 8.44^{b}	44.0 ± 4.38^{c}	92.5 ± 5.02^{d}	-
AGR (% body weight gain/day)	21.1 ± 4.70^{a}	8.40 ± 3.07^{b}	7.97 ± 0.77^{b}	19.5 ± 2.18^{a}	0.15 ± 0.51^{c}
NPU (%)	36.6 ± 3.47^{a}	18.6 ± 2.14^{b}	20.4 ± 2.03^{b}	38.6 ± 2.10^{a}	-
PER	2.37 ± 0.22^{a}	1.17 ± 0.14^{b}	1.35 ± 0.13^{c}	2.46 ± 0.13^{a}	-

Note: Numbers in the same row with different superscripts are significantly different ($P<0.05$). Values are mean ± standard deviation of three replicates (N = 3)

Table 4-4 Protein intake, protein efficiency ratio (PER), average growth rate (AGR) and survival rate of shrimps fed for 3 weeks with different food combinations (pellets + chironomid larvae or mosquito larvae at 50:50 or 25:75, or with 100% pellets as control)

	100% pellets (Control) (T6)	50% pellets + 50% chironomid larvae (T7)	25% pellets + 75% chironomid larvae (T8)	50% pellets + 50% mosquito larvae (T9)	25% pellets + 75% mosquito larvae (T10)
Protein intake (g wet weight/g shrimp/day)	0.043 ± 0.000^{a}	0.043 ± 0.000^{a}	0.043 ± 0.000^{a}	0.043 ± 0.000^{a}	0.042 ± 0.000^{a}
PER	2.59 ± 0.21^{ab}	3.05 ± 0.30^{a}	2.88 ± 0.19^{ab}	2.79 ± 0.28^{ab}	2.55 ± 0.25^{b}
AGR (% body weight gain/day)	26.2 ± 1.97^{a}	34.1 ± 1.44^{b}	30.6 ± 3.43^{ab}	28.5 ± 3.26^{a}	25.3 ± 3.39^{a}
Survival rate (%)	86.7±2.89	83.3 ± 12.6	78.3 ± 5.77	85.0 ± 0.00	78.3 ± 5.77

Note: Numbers in the same row with different superscripts are significantly different ($P<0.05$). Values are mean ± standard deviation of three replicates (N = 3)

CHAPTER V

STIMULATION BY GUTWEED TO INCREASE THE ABUNDANCE OF INSECT LARVAE AS FOOD FOR SHRIMP AQUACULTURE

5.1 Introduction

From the results in Chapters III and IV, the importance of insect larvae in shrimp aquaculture ponds was elucidated and the way to stimulate the occurrence of these insect larvae in ponds will be investigated in this chapter.

Gutweed, *Ulva intestinalis*, is a widely distributed green alga which grows in some eutrophic shrimp ponds in Thailand (Lewmanomont and Ogawa, 1995). Gutweed could, therefore, increase nutrient uptake and reduce the concentration of inorganic nutrients in shrimp ponds. In addition, gutweed may provide the shelter for insect larvae in the natural and aquaculture ponds.

The objective of this chapter is to evaluate the stimulatory effects of different amounts of gutweed on the occurrence of insect larvae and the consequent growth of shrimps when gutweed was planted in shrimp ponds. This knowledge will be useful for reducing aquaculture costs by replacing expensive pellet feed, to some extent, with naturally-occurring insect larvae. It is also beneficial to guide shrimp farmers in the preparation of ponds to achieve a high abundance of nutritional natural food and to promote environment-friendly shrimp aquaculture.

5.2 Materials and methods

Occurrence of insect larvae with no shrimp present (Experiment 1)

Preparation of experimental tanks

Tank-scale experiments were carried out at Coastal Aquaculture Research and Development Regional Center 6 (Songkhla), Songkhla Province, in Thailand. The experiment was conducted by a completely randomized design (CRD) of three treatments, with three replicates for each. The three treatments were as follows: no gutweed planted (T1, control), gutweed planted at 28 g/m^2 (T2) and gutweed planted at 56 g/m^2 (T3).

The experiment consisted of nine units of brackish water microcosms (in fiber-glass cylinder tanks, surface area 0.71 m^2, height 50 cm and volume 350 l), to which the sludge from a shrimp pond (depth 10 cm) and 35.5 liters of brackish water of 20 psu salinity (depth 5 cm) were added. The sludge was raked once a week for two weeks in order to enhance the process of oxygen transfer and nutrient release. After 2 weeks, more brackish water was added and the water depth was adjusted to 10 cm, then gutweed was planted in treatments T2 and T3 and allowed to grow for a further 2 weeks.

Other pieces of gutweed (3 g, wet weight) were put in baskets, three of which were hung in each microcosm (T2 and T3). The biomass of gutweed in each basket was weighed weekly, and the total biomass of gutweed in each microcosm was calculated by the following equation:

$$WG \text{ (g/pond)} = (WS_t / WS_0) \times WP_0$$

Where: WG = Weight of gutweed (g/pond), WS_0= Initial weight of gutweed in the basket (g), WS_t = Weight of gutweed in basket at t days (g), WP_0 = Initial weight of gutweed in the pond (g)

Observation of insect larvae

Subsamples (3 liters) of tank water were collected from each microcosm weekly and filtered through a net of 22 µm mesh size. The solid samples recovered were

preserved in 10% formalin for subsequent investigation of the insect larvae in the water column.

Sediment was also collected from an area of 200 cm^2 (5 cm depth) weekly and solid material was collected in a sieve with 420 µm pore size. The sampling area of sediment in a tank was divided into six areas, one of which was randomly sampled in one tank weekly. Samples were preserved in 10% formalin and used for identification of insect larvae.

Pieces of gutweed (3 g, wet weight) were collected from the surface, middle and bottom of the water column in each microcosm because gutweed grew throughout the water column. Samples were preserved in 10% formalin and used for observation of insect larvae.

Shrimp growth and occurrence of insect larvae (Experiment 2)

Preparation of experimental tanks

Three feeding regime treatments, without pellets (T4, control), with commercial pellet feed (T5), and without pellets but with gutweed planted (27 g/m^2 for two weeks) (T6) were designed, with three replicates of each.

The experiment consisted of nine units of brackish-water microcosms (2.25 m^2, depth 80 cm and volume 1,800 l) to which the sludge from a shrimp pond (depth 10 cm), and 125 liters (depth 5 cm) of brackish water (salinity 16 psu.) were added. The sludge was raked once a week for two weeks at the first day and seventh day of the experiment in order to enhance the process of oxygen transfer and nutrient release. After 2 weeks, gutweed was planted into the ponds and allowed to grow for 2 more weeks as in Experiment 1. Then postlarvae of black tiger shrimp *(Penaeus monodon)* (average weight

0.013 g/ind.) were stocked into each microcosm at a density of 53 individuals/m^2 and cultured for 5 weeks.

Shrimp growth and insect larvae count

Ten shrimps were randomly sampled every week from each microcosm. The shrimps were weighed, and the average body weight was calculated. After weighing, living shrimps were returned to the ponds. The observation of insect larvae in the water column, the bottom sediment and attached to gutweed were same as for Experiment 1.

5.3 Results

Gutweed biomass and the numbers of insect larvae (Exp.1)

Gutweed in the tanks grew throughout the water column, from bottom to surface water. The small thallus of gutweed (which has a small hole) sank to the bottom sediment. When it grew, some thallus attached to the bottom sediment and some extended in size and had a bigger hole, so it floated to the surface water. The biomass of gutweed increased continuously until the end of the experiment. At the end of the experiment, the average of gutweed biomass of T2 (28 g/m^2 planted) was 484 ± 508 g/m^2 and that of T3 (56 g/m^2 planted) was 1,250 ± 1,063 g/m^2 (data not illustrated), which were 17.2 (T2) and 22.2 (T3) times greater than those of the initial biomass, respectively.

In the fiber-glass tanks without shrimps, a few chironomid larvae were already present in week 0 when gutweed was planted (Fig. 5-1a). After that, the numbers of total chironomid larvae increased continuously. In weeks 2 and 3, the numbers of chironomid larvae in treatment T3 with the high level of gutweed planted was higher than those in T2 and the control (T1). Figure 1b shows the % occurrence of chironomid larvae in T3. Along

with increasing numbers of chironomid larvae, high percentages of chironomid larvae were associated with the gutweed (Fig. 5-1b).

Mosquito larvae were found in the tank before gutweed was planted (week -1) in all treatments and their number increased continuously until the end of the experiment (Fig. 5-2a). In contrast to the chironomid larvae, few mosquito larvae were associated with the gutweed (Fig. 5-2b).

Analysis showed that the number of insect larvae occurring in the microcosm was related to the level of gutweed biomass (Fig. 5-3). The correlation between chironomid larvae and the gutweed biomass was particularly high (Fig. 5-3a)

Growth of shrimps and insect larvae (Exp. 2)

The shrimps, with an average initial weight of 0.013 g/individual, were grown for 5 weeks, and the final average body weight was 0.411 ± 0.020 g/individual (T4, no food as control), 0.976 ± 0.285 g/individual (T5, with pellets), and 0.635 ± 0.205 g/individual (T6, no pellets added but with gutweed planted), respectively (Fig. 5-4). The average body weight of shrimps in both T5 and gutweed-planted (T6) tanks was not significantly different ($P>0.05$) but was significantly higher ($P<0.05$) than that in the control (T4) in week 4. At the end of the experiment, the average body weight of shrimps in T6 (the gutweed-planted ponds) was not significantly different ($P>0.05$) from that in T5.

Chironomid larvae were present in the second week of the experiment (week -2), and their number then increased in all treatments (Fig. 5-5a). After release of shrimp larvae into the tanks, the number of chironomid larvae increased continuously in the gutweed-planted tanks (T6) and also slightly increased in the pellet-feeding tanks (T5)

but decreased in the control (T4). After 2 weeks of shrimp culturing, the number of chironomid larvae decreased substantially in all treatments (Fig. 5-5a).

Mosquito larvae occurred in the first week of the experiment (week -3) (Fig. 5-5b). There were large fluctuations in the numbers of mosquito larvae in all treatments (Fig. 5-5b). In week 2 of shrimp culturing, numbers of mosquito larvae in T5 and T6 were higher than those in the control (T4). After 3 weeks of shrimp culturing, the number of mosquito larvae decreased in all treatments, but the number of mosquito larvae in the gutweed-planted tanks (T6) was higher than in the control (T4) which continuously decreased to nearly zero in week 4 (Fig. 5-5b).

5.4 Discussion and conclusion

Shrimp ponds accumulate organic matter in the bottom sediment (Funge-Smith and Briggs, 1998; Avnimelech and Ritvo, 2003) and insect larvae mainly consume organic matter (Hirabayashi and Wotton, 1999; Malmqvist et al., 2001). Results from previous chapters indicated that insect larvae are an important natural food for cultured shrimps, both under natural conditions and also in aquaculture ponds. The stomach content of shrimps living in the natural environment or in aquaculture ponds contained much debris of insect larvae (Martínez-Córdova and EnríquezOcaña, 2007; Muangyao et al., 2011; Varadharajan and Pushparajan, 2013). Insect larvae have a high protein content and are predicted to be an alternative protein source for aquaculture (Panini et al. 2017a,b; Belghit et al., 2018; Concetta Elia et al., 2018; Henry et al. 2018; Iaconisi et al. 2018; Sankian et al. 2018). They are acceptable to shrimps and can be digested very quickly (Chapter III, Muangyao et al., 2019a). Shrimps were able to convert protein from the insect larvae, especially chironomid larvae, into the shrimp body at a level as high as that

from pellets (Chapter IV, Muangyao et al., 2019b). This should provide a valuable nutrient resource, especially if the occurrence of insect larvae in the shrimp pond can be stimulated.

Some studies have reported that gutweed can grow in supralittoral rock pools which have extreme environmental variation, and that the hollow thallus can act as a refuge for organisms such as copepods and chironomid larvae (McAllen, 1999). A previous study (Suriyaphan et al., 2008) showed that the number of benthic organisms in shrimp ponds was higher with gutweed present than without gutweed. This indicates a probable benefit of using gutweed to enhance the numbers of insect larvae in shrimp ponds and reduce the nutrient concentration. Chironomid larvae were found in raked shrimp-culturing microcosms after two weeks (Fig. 1a), whilst mosquito larvae were present in the first week in all treatments (Fig. 2b). Many aquatic insects, whose larvae develop in water, are associated with water (Schwind, 1991, 1995). Adult insects will lay eggs on the water surface or on the substratum near or above the water level. Eggs of chironomids hatch within 2-5 days after they are laid (Soong et al., 1999; Zilli et al., 2009). Newly hatched larvae remain free-living for one day to a few days before they settle on the substrate, whilst mosquito eggs hatch into larvae within 48 hours then live in the water (American Mosquito Control Association: http://www.mosquito.org/life-cycle/"Accessed 7 Nov 2018".). In a marsh which has a high concentration of organic matter, the insects are induced to lay eggs into that area.

The number of insect larvae is related to the biomass of gutweed planted. Higher biomass of gutweed stimulated more the growth of chironomid larvae (Fig. 5-1a) and percentages of chironomid larvae attached to gutweed increased along with increasing gutweed biomass (Fig. 5-1b). Chironimid larvae are more highly associated with gutweed

than are mosquito larvae. Chironomid larvae are benthic organisms; the larvae will settle and use small pieces of debris and fine sand to build nest-tubes on the surface or in crevices of dead coral skeleton or other substrates (Soong et al., 1999). So gutweed may play an important role as substratum or shelter to which chironomid larvae could attach and settle into the thallus where they can find plant debris and particulate matter. Mosquito larvae, depending on the species, can live in different sections of the pond, i.e. surface, water column and also on the bottom sediment, but they spend most of their time feeding on microalgae, bacteria etc. in the surface microlayer (Wikipedia: https://en.wikipedia.org/wiki/Mosquito "Accessed 30 Dec 2018".). The observation data about mosquito larvae showed that most of them were free-living and usually came to the surface water and stayed close to the thallus of gutweed, though only a few were attached to this. They escaped from that area, however, when they were disturbed, including by the sampling of gutweed, so samples of gutweed had few mosquito larvae (Fig. 5-2b). This indicates that the practice of raking sediment and planting gutweed stimulates the numbers of insect larvae, especially of chironomid larvae.

The shrimps could grow for 4 weeks in gutweed-planted tanks (T6) without any pellet food (Fig. 5-4). The number of insect larvae in the gutweed-planted tank (T6) is, therefore, enough for the shrimps at least for 4 weeks. Within three weeks, however, growth of the shrimps was not significantly different in all treatments (Fig. 5-4). This suggests that raking the sediment in order to release organic matter could stimulate the occurrence of enough insect larvae to support shrimp growth for up to 3 weeks, but after 3 weeks, gutweed planting was necessary in shrimp ponds.

Shrimp culturing tanks where gutweed was planted had high numbers of both chironomid larvae and mosquito larvae (Fig. 5-5), both of which have high protein

contents of about 62% dry matter, similar to that of fishmeal (Chapter IV). This indicated that shrimps consumed insect larvae, which were easily digested, and converted insect larvae protein into that of their own body; about 36% of the protein in chironomid larvae could be converted into shrimp body protein. This value was higher than that of mosquito larvae protein, and comparable to that of pellet feed protein. Furthermore, the combination of pellets and larvae can be more effective than feeding either pellets or larvae alone; up to 75% of pellet feed could be replaced by the insect larvae without any negative effect on shrimp growth. The chironomid larvae in a shrimp pond will support more shrimp growth than can be achieved simply by feeding pellets alone. Insect larvae are considered interesting for use as nutrient sources for fish aquaculture (Lock et al., 2016; Belghit et al., 2018; Concetta Elia et al., 2018; Vargas-Abúndez et al., 2019). Some species of insect larvae have shown great potential as protein and oil sources (Belghit et al., 2018).

The present study showed that, after release of the shrimp larvae, the number of chironomid larvae in shrimp-culturing tanks where gutweed was planted (T6) continued to increase until after 1 week while that in control (T4) decreased immediately after shrimp culturing (Fig. 5-5a). In addition, although after 2 weeks of shrimp culturing, the number of chironomid larvae decreased substantially to nearly zero in all treatments (Fig. 5-5a), numbers of chironimid larvae in the tanks without shrimps still increased in all treatments (Fig. 5-1a). These results show that the chironomid larvae were consumed by shrimps. In contrast to this, the number of mosquito larvae still increased until 2 weeks after shrimp were released (Fig. 5-5b). This confirmed the finding in a previous study (Chapter III) that shrimps prefer chironomid larvae to mosquito larvae. The numbers of mosquito larvae decreased after 3 weeks had passed from the time shrimps were released,

in all treatments (Fig. 5-5b). The number of mosquito larvae in the gutweed-planted tanks (T6), however, was higher than in the control (T4) after the 3rd week, but then decreased to nearly zero. This is consistent with the finding that the shrimps could grow for 4 weeks in the gutweed-planted tank but only for 3 weeks in the control tank.

In conclusion, the results obtained in this study confirmed the importance of insect larvae, especially chironomid larvae, for the growth of shrimps and they confirmed the role of gutweed in stimulating the abundance of insect larvae. During preparation of a shrimp pond to use organic matter as a nutrient source for insect larvae, putting only a little water into the pond at first, to give a water depth of 15-30 cm, and raking the bottom sediment to resuspend a high concentration of organic matter will enhance the density of insect larvae. This treatment would also reduce the impact of waste from shrimp culture on the environment. Enhancing the population of insect larvae in a shrimp pond, therefore, can reduce the amount of expensive protein-rich feed that needs to be supplied, thereby reducing the cost of producing cultured shrimps

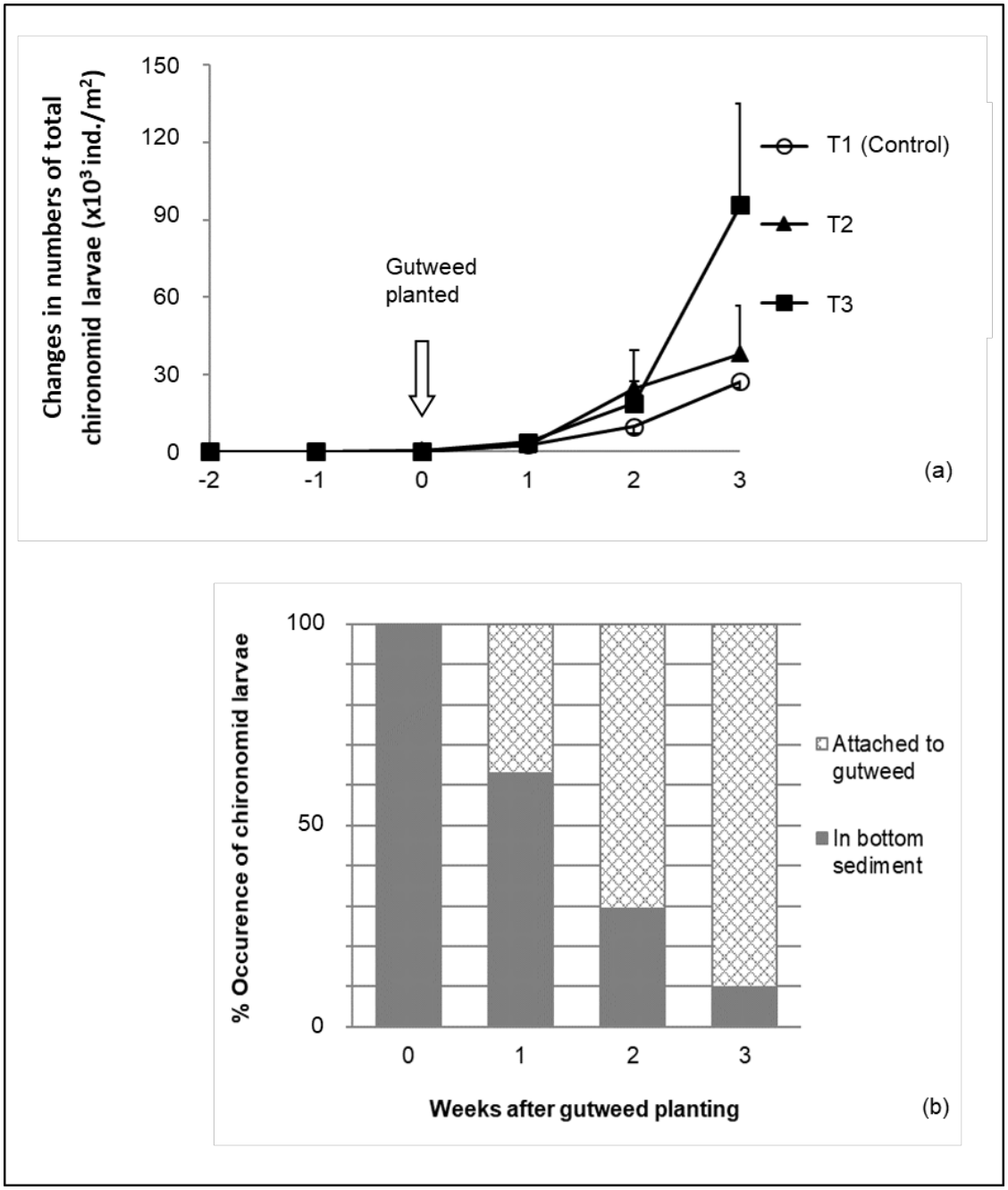

Fig. 5-1 Changes in numbers of total chironomid larvae in microcosms without gutweed planted (T1), stimulated by planting gutweed at 28 g/m^2 (T2), or at 56 g/m^2 (T3) (a), and the percentage of larvae attached to gutweed and in the bottom sediment in the T3 tank (b) observed over 3 weeks**.**

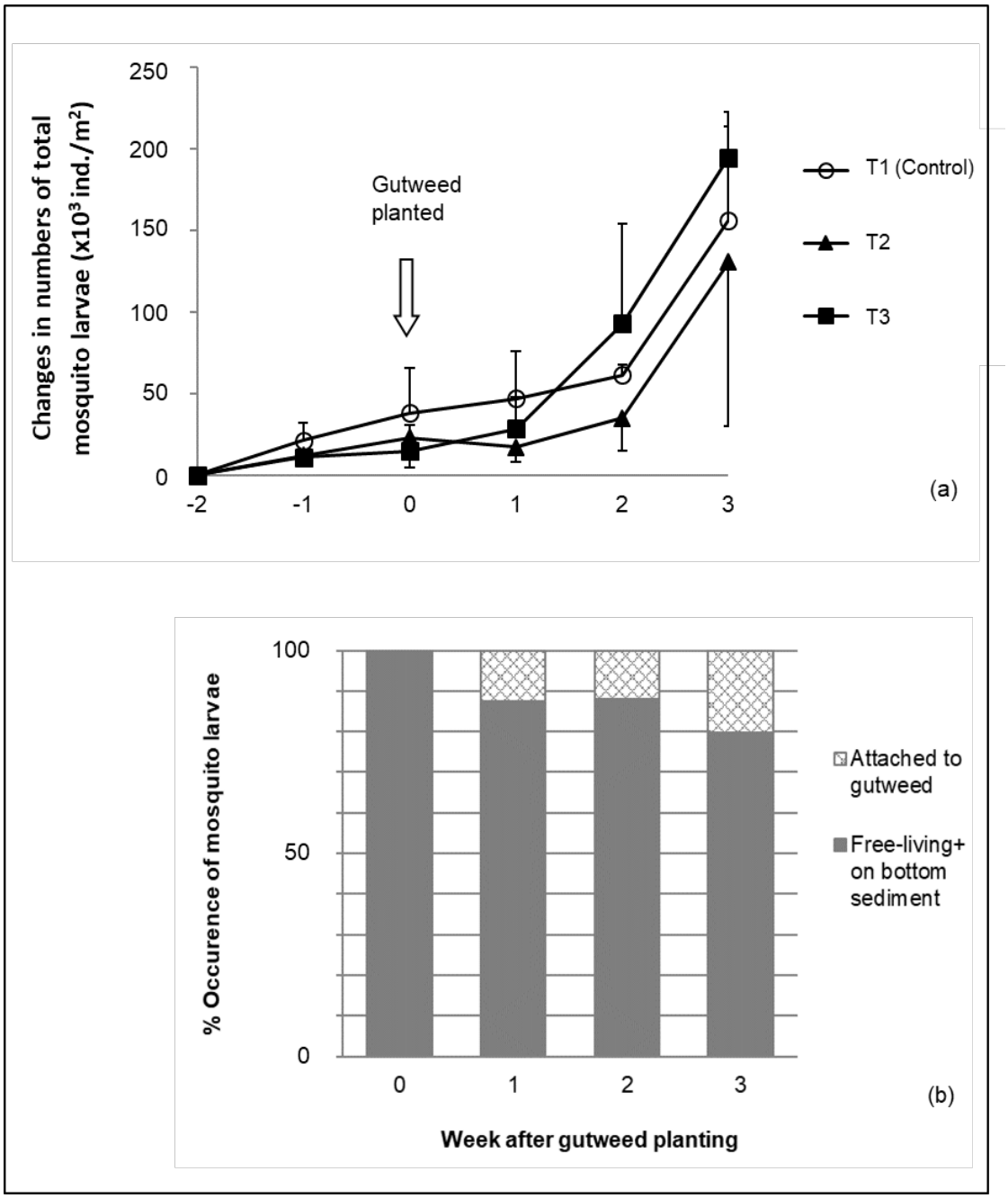

Fig. 5-2 Changes in the number of mosquito larvae in microcosms with no gutweed planted (T1), stimulated by planting gutweed at 28 g/m^2 (T2), or at 56 g/m^2 (T3) (a), and the percentage of larvae attached to gutweed, and free-living and in the bottom sediment in the T3 tank (b) observed over 3 weeks.

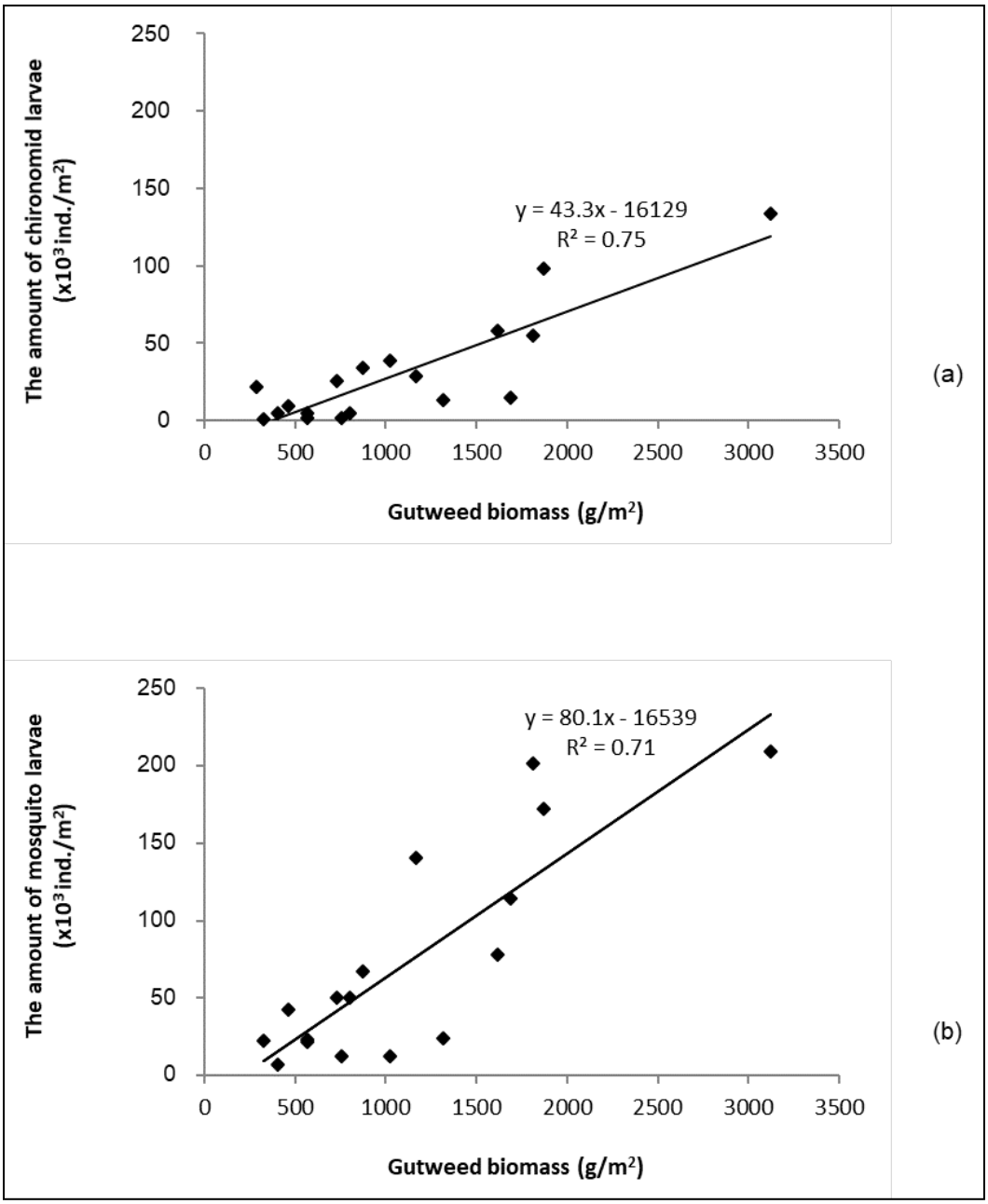

Fig. 5-3 Relationship between the amount of gutweed planted and the numbers of chinoromid larvae (a) and mosquito larvae (b) in gutweed planted microcosms.

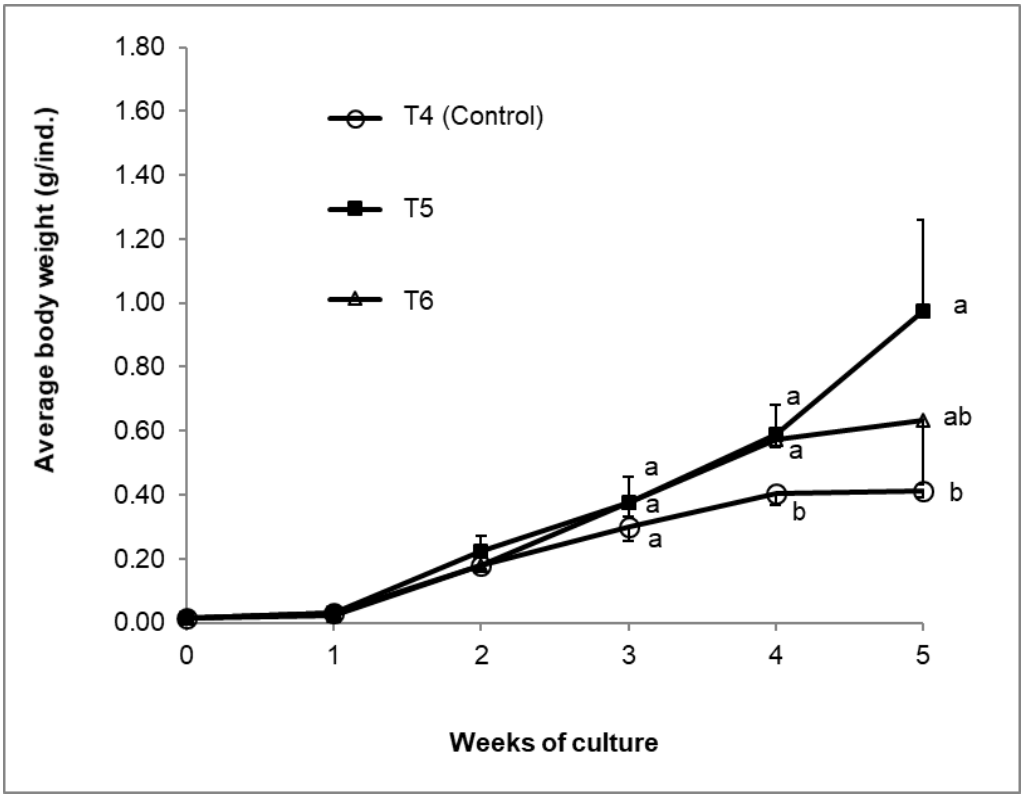

Fig. 5-4 Growth of shrimps reared for 5 weeks, with pellet (T5), and with gutweed (T6) compared with the control (T4).

Note: Values in the same weeks of culture with different letters are significantly different ($P<0.05$).

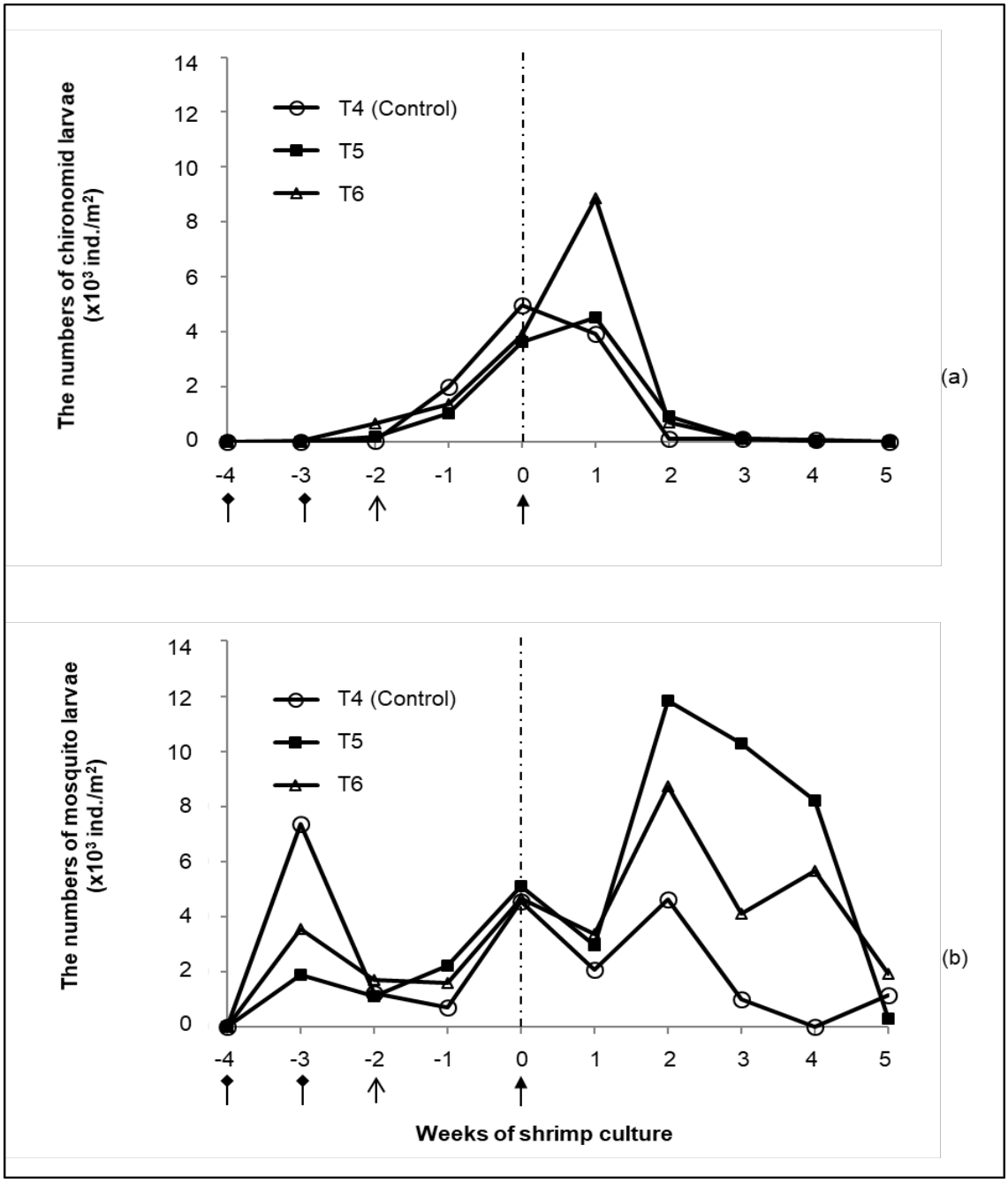

Fig. 5-5 The numbers of chironomid larvae (a) and mosquito larvae (b) in ponds during the pond preparation period and the shrimp culture period with pellets (T5), or with gutweed (T6) compared with the control (T4).

Note: The arrow ↑ indicates the time of sediment raking, the arrow ↟ indicates the time of gutweed planting and the arrow ↑ indicates the time of shrimp release.

CHAPTER VI

TRANSFORMATION OF NITROGEN IN SHRIMP PONDS WITH DIFFERENT FEEDING REGIMES

6.1 Introduction

In previous chapters it was shown that chironomid larvae are easily digested by shrimps and were the most acceptable natural food provided. An abundance of chironomid and mosquito larvae in a shrimp pond can support growth of juvenile shrimps more than simply feeding pellets alone. The role of gutweed in stimulating the abundance of insect larvae in shrimp ponds was also clarified.

The aim of this chapter is to estimate the transformation of nitrogen in white shrimp culture ponds using microcosms in which the cycling of nutrients is stimulated by planting gutweed. This would be of benefit by improving pond preparation practice in a way that would support shrimp growth, reduce the need for pellets and minimize negative effects of organic burden by feeding on the natural environment, thereby supporting eco-friendly shrimp aquaculture.

6.2 Materials and methods

Preparation of experimental microcosms

The experiments were carried out at the Coastal Aquaculture Research and Development Regional Center 6 (Songkhla), Songkhla Province, in Thailand, by a completely randomized design (CRD) of four feeding regime treatments, with duplicates for each. The four treatments were as follows: without pellets (as a control, T1), with

commercial pellet feed (T2), without pellets but with gutweed planted (25 g/m^2 for three weeks) (T3) and with pellets and with gutweed planted (25 g/m^2 for three weeks) (T4).

The experiment consisted of eight units of brackish water microcosms (in plastic tanks, area 3 m^2, height 80 cm and volume 2,400 L), to which the sediment from a shrimp pond (depth 10 cm) and 300 liters of brackish water of 20 psu salinity (depth 10 cm) were added. The sediment was raked once a week for two weeks, the water depth was adjusted to 20 cm at the 3rd week by addition of more brackish water, and then gutweed was planted in treatments T3 and T4 and allowed to grow for 3 more weeks. Postlarvae of white shrimp *(Litopenaeus vannamei)* (average weight 0.003 g/ind.) were then stocked into each microcosm at a density of 50 individuals/m^2 and cultured for 5 weeks (See Chapter II for details).

Three baskets, each of which contained other pieces of gutweed (3 g, wet weight), were hung in each microcosm (T3 and T4). The biomass of gutweed in each basket was weighed weekly, and the total biomass of gutweed in each microcosm was calculated (See Chapter V for details).

Determination of nitrogen assimilation by phytoplankton and gutweed

The uptake (assimilation) of nitrogen compounds by gutweed was determined by collecting water samples from each microcosm, filtering them through GF/C filter paper, putting into the BOD bottle (300 L in volume) and adding gutweed (1.0 g). To determine the assimilation of nitrogen compounds by phytoplankton, water samples were taken from each of the experimental microcosms and put into a BOD bottle without filtering through GF/C filter paper. In both cases, BOD bottles with filtered water without gutweed nor phytoplankton were used as controls. In all cases, the BOD bottle was closed tightly, and

the bottles were returned in the experimental microcosms and incubated for 1 day (24 hours). After this, the water in each bottle was filtered through GF/C filter paper and stored at -20°C before analysis of the total dissolved nitrogen compounds (TDN, mg/L). The values obtained were used to calculate the assimilation rate of nutrients as follows:

$$\text{Nitrogen assimilation rate of gutweed (g-N/g Gutweed/day)} = \frac{(\text{TDN in control bottle} - \text{TDN in gutweed bottle}) \times \text{Vol. of BOD bottle (mL)} \times \text{Gutweed weight (g)}}{1000}$$

and

$$\text{Nitrogen assimilation rate of phytoplankton (g-N/day)} = \frac{(\text{TDN in control bottle} - \text{TDN in non-filtered bottle}) \times \text{Vol. of BOD bottle (mL)}}{1000}$$

Determination of shrimp biomass and of nitrogen in water, shrimp, phytoplankton, gutweed and sediment

The body weights of shrimps were determined, and numbers of living shrimps were counted at the end of the experiment. The biomass of shrimps was calculated and the content of organic nitrogen in the shrimp body was determined. Samples of shrimp and gutweed were freeze-dried and the samples of sediment were oven-dried; all were ground before the organic nitrogen content was analysed by CHN analyser (Truspec CN, LECO). The total nitrogen and total dissolved nitrogen in the water samples were analysed by the persulfate oxidation method (Hansen and Koroleff, 1999).

The total nitrogen in water and sediment was determined at the beginning and the end of the feeding experiments. The values of nitrogen input by shrimp, water, and sediment (at the beginning of the experiment) and of output by shrimp, water, and sediment (at the end of experiment) were calculated.

The phytoplankton nitrogen (mg/L) was then calculated according to the linear equation, $Y = aX + b$, between the relationship of Particulate organic nitrogen (Y) and Chlorophyll a content (X).

The equation is Particulate organic nitrogen (mg/L) = 0.0054 × Chlorophyll a (ug/L) + 0.2285 ($r^2 = 0.61$)

Where, a is linear coefficient that is multiplied by Chlorophyll a content to give phytoplankton nitrogen (mg/L)

b (0.2285) is the constant that represents the non-phytoplankton nitrogen

Nitrogen budget calculations

A static model was used to calculate nitrogen (N) budget into and out of subunits in shrimp microcosms. The concept of a balanced budget of the nitrogen (g-N/microcosm), is that nitrogen entering (input) is equal to the nitrogen leaving (output). There was one balance equation for each subunit. In each balance equation, there was a measurable nitrogen process (for which the amount of nitrogen was measured in this study) and there was one un-measurable nitrogen process that was estimated by balancing the equation in each subunit. This gave an estimated value.

The static model for the balanced budget of nitrogen in white shrimp microcosms consists of 5 subunits, namely phytoplankton subunit, gutweed subunit, water subunit,

shrimp subunit and sediment subunit. The quantity of nitrogen in each process that flows through each subunit was assessed by measuring the amount of nitrogen entering at the beginning (nitrogen input) of the culture period and leaving each subunit (nitrogen output) at the end of the operation (Fig. 6-1). The balance equation for each subunit, name of process (number of process), and the equation to calculate the nitrogen in each process of each subunit are as follows;

Phytoplankton subunit

The nitrogen balance equation of the phytoplankton subunit is

(1) + (3) = (2) + (4),

where (4) is an un-measurable nitrogen process and

N phytoplankton input (1)	=	Phytoplankton nitrogen (mg/L) × Water volume at the beginning of experiment and added during experiment (L)/1000
N phytoplankton output (2)	=	Phytoplankton nitrogen (mg/L) × Water volume at the end of experiment (L)/1000
N phytoplankton assimilation (3)	=	TDN absorption rate (g/L/day) × Experimental period (day) x Water volume (L)
N phytoplankton sink (4)	=	[(1) + (3)] - (2)

Note: N phytoplankton sink (4) is nitrogen in decayed phytoplankton that sinks to the sediment. This part of the nitrogen is estimated as (1) + (3) - (2).

Gutweed subunit

The nitrogen balance equation of the gutweed subunit (calculated only in T3 and T4) is

(5) + (7) = (8) + (9),

where (9) is an un-measurable nitrogen process and

N gutweed input (5)	=	Gutweed at the beginning of experiment (g) × TN content in gutweed (% wet matter)
N gutweed assimilation (7)	=	Gutweed weight (g) × TDN assimilation rate (g/g gutweed/day) × Experimental period (day)
N gutweed output (8)	=	Gutweed weight at the end of the experiment (g) × TN content in gutweed (% wet matter)
N gutweed sink (9)	=	[(5) + (7)] – (8)

Note: N gutweed sink (9) is nitrogen in decayed gutweed that sinks to the sediment. This part of the nitrogen is estimated as [(5) + (7)] – (8).

<u>Water subunit</u>

The nitrogen balance equation of the water subunit is

(10) + (11) + (13) + (19) = (3) + (7) + (12),

where (13) is an un-measurable nitrogen process and

N water input (10)	=	Water volume at the beginning of experiment (L) × TN concentration in water (g/L)
N water addition (11)	=	Water volume added during the experiment (L) × TN concentration in water (g/L)

N water output (12)	=	Water volume at the end of experiment (L) × TN concentration in water (g/L)
N (shrimp excretion + solubilizing from the sediment) (13)	=	[(3) + (7) + (12)] – [(10) + (11)]
N sediment raking (19)	=	TN increase after sediment raking (g/L) x Water volume in pond (L)

Shrimp subunit

The nitrogen balance equation of the shrimp subunit is

(14) + (15) + (18) = (16) + (17),

where (18) is an un-measurable nitrogen process and

N shrimp input (14)	=	Total shrimp weight at the beginning of experiment (g) × TN content of shrimp (% wet matter)
N feed input (15)	=	Total pellets feed (g) × TN content of pellets (% as fed)
N shrimp output (16)	=	Total shrimp weight at the end of experiment (g) × TN content of shrimp (% wet matter)
N shrimp excretion (17)	=	(N shrimp output (g) × 100/25 × (75/100) (Nitrogen excretion in shrimp is 75% of nitrogen eaten, Burford and Lorenzen, 2004)
N natural productivity (18)	=	[(16) + (17)] – [(14) + (15)]

Sediment subunit

The nitrogen balance equation of the sediment subunit is

(4) + (6) + (9) + (21) = (18) + (19) + (20) + (22) +(23),

where (6) or (23) is an un-measurable nitrogen process and

N insect larvae contribution (6)	=	[(18) + (19) + (20) + (22)] - [(4) + (9) + (21)]
N solubilizing from the sediment (20)	=	(13) – (17)
N sediment input (21)	=	Sediment input (g) × TN content in sediment (g/g)
N sediment output (22)	=	Sediment output (g) × TN content in sediment (g/g)
N denitrification (23)	=	[(4) + (9) + (21)] – [(18) + (19) + (20) + (22)]
Net N accumulation (24)	=	Total nitrogen in sediment at the end of experiment (g) – Total nitrogen in sediment at the initial (g)

The transformation of nitrogen in the sediment subunit is complicated. The denitrification process was used for the balance equation of this subunit. This is net nitrogen in the sediment subunit and is an estimated value. The N denitrification (23) or N insect larvae contribution by gutweed plantation (6) are the same value (only in T3 and T4). When the budget of the sediment subunit was balanced and if the value indicated flow out of sediment, this value will be N denitrification (23). But if this value indicated flow into sediment, this value will be N insect larvae contribution by gutweed plantation (6). The N insect larvae contribution (6) process occurred in T3 and T4 and the value in T4 was calculated as a proportion compared to gutweed biomass in T4.

6.3 Results

Nitrogen budget

The nitrogen budgets in T1 (control), T2, T3 and T4 are shown in Figs 6-2, 6-3, 6-4 and 6-5, respectively (Table 1 in Appendix). The nitrogen input from water (10), water addition (11), phytoplankton (1), shrimp (14) and sediment (21) was similar in each treatment. The input from gutweed (5) in T3 and T4 was equal (Figs. 6-4, 6-5). The nitrogen was released by raking the sediment (19). The phytoplankton assimilation (3) was higher than the gutweed assimilation (7) in all treatments. The nitrogen in shrimp output (16) was highest in T4 (with both pellet and gutweed) (Fig. 6-5), followed by T2 (with pellet) (Fig. 6-3), T3 (without pellet, with gutweed) (Fig. 6-4) and T1(control) (Fig. 6-2), respectively. The nitrogen in water output (12) was highest in T4 (Fig. 6-5), followed by T3 (Fig. 6-4), T2 (Fig. 6-3) and T1 (Fig. 6-2), respectively. The nitrogen in phytoplankton output (2) was highest in T2, followed by T4, while there was only a little in T1 and T3. Similarly, the nitrogen in sediment output (22) was highest in T2 (Fig. 6-3). The solubilization from the sediment (20) showed that sediment absorbed nitrogen in the microcosms with pellet feeding (T2 and T4), whilst the sediment released nitrogen in the microcosms without pellet feeding. Thus, the highest accumulation of nitrogen in sediment was in microcosms with pellet feeding but without gutweed planted (T2) (Fig. 6-3), whilst the shrimp microcosms with pellet feeding and gutweed planted (T4) had net accumulation of nitrogen (24) in sediment of only 0.290 g-N. For the control (T1), the nitrogen in the sediment output (22) was comparable to or a little lower than the nitrogen in the sediment input (21), so nitrogen did not accumulate in sediment in this treatment.

In microcosms in which gutweed was planted (T3, T4), insect larvae (6) also contributed nitrogen; the contribution in T4 was higher than in T3 (Figs. 6-4, 6-5). The

insect larvae + natural productivity in the microcosm T4 had the highest amount, followed by T2, T3 and T1, respectively (Fig. 6-6).

Survival rate and biomass of shrimps

The survival rate of shrimps was highest in T4 (with both pellet and gutweed) and lowest in T1 (control), but these values were not significantly different in all treatments ($P>0.05$) (Table 6-1). The biomass of shrimps, however, was highest in T4 and this was significantly higher than that in other treatments (Table 6-1). The biomass of shrimps was lowest in T1 (control). This was not significantly different from that in T3 ($P>0.05$) but higher than that in T2 ($P<0.05$) (Table 6-1).

6.4 Discussion and conclusion

This study was carried out to understand the transformation of nitrogen in shrimp ponds with different feeding regimes, including understanding the role of gutweed on transformation of nitrogen from sediment through naturally-occurring insect larvae to shrimp biomass. Raking the sediment released nutrient (nitrogen) to the water subunit, and this was transferred to primary productivity (phytoplankton and gutweed) by assimilation. The primary productivity was enhanced by suitable water quality and affected the water quality in the shrimp pond (Haque and Rahman. 2008), in turn enhancing secondary and tertiary production. Insect larvae contributed to this pathway in the shrimp pond with planted gutweed and their contribution was positively related to the gutweed biomass. The observation data showed that there was spawning of insects, especially mosquitoes, on the surface of the water (data not illustrated) and confirmed the finding in chapter V that the number of insect larvae was related to the biomass of gutweed planted. Shrimps fed on pellets in the microcosm with planted gutweed (T4) had

the highest natural productivity from insect larvae (Fig. 6-5, 6-6). Furthermore, this gave the highest survival rate and higher shrimp biomass (Table 6-1) when compared to microcosms in which shrimps were fed pellets but no gutweed was planted. These results were consistent with the role of gutweed as a shelter in shrimp ponds (Muangyao et al., 2019c). Consumption of natural and artificial foods together by white shrimp (*Litopenaeus vannamei*) leads to better growth of shrimps reared in ponds with and without enhancement of natural productivity (Porchas-Cornejo et al., 2012).

Shrimp ponds with pellet feeding lead to a high concentration of nitrogen in water and sediment, the quality of which plays an important role in increasing the productivity of ponds and provides a nutritionally balanced and healthy environment for the cultured animals (Kumar et al., 2012). The pond with pellet feeding had the highest nitrogen accumulation in sediment, thus giving poor quality of environment and could poison shrimps living in the pond. All intensive shrimp ponds have problems with sludge formation on the pond bottom and this directly affects the quality of water in shrimp farming (Chanratchakool et al., 1998). Shrimps normally live on or near the bottom, and are exposed and respond to the conditions of the sludge. Results obtained in this chapter showed that when gutweed was planted, however, the concentration of nitrogen in water and sediment was lower and the accumulation was eight times lower than in microcosm in which pellets were fed but no gutweed was planted (Figs. 6-3 and 6-5).

In conclusion, raking sediment stimulates the release of nutrients (nitrogen) to the water column and into primary productivity, thus enhancing the nitrogen cycling in shrimp ponds. The planting of gutweed stimulates the transformation of nitrogen through

insect larvae and natural productivity. This is expected to reduce the accumulation of nitrogen in sediment and to promote an eco-friendly system for shrimp aquaculture.

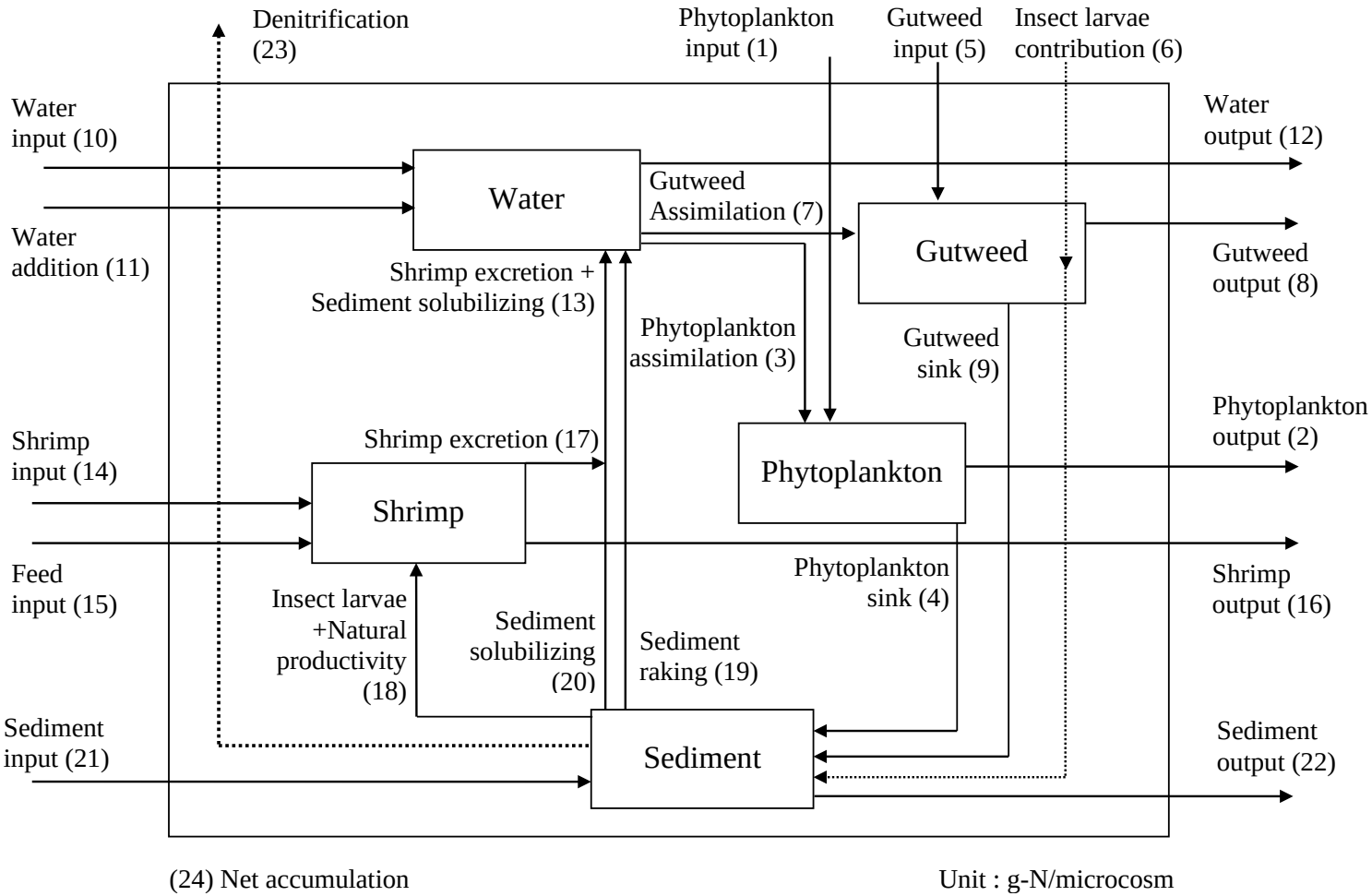

Fig. 6-1 A model of the transformation of nitrogen in shrimp microcosms consisting of 5 subunits, namely water, phytoplankton, gutweed, shrimp and sediment.

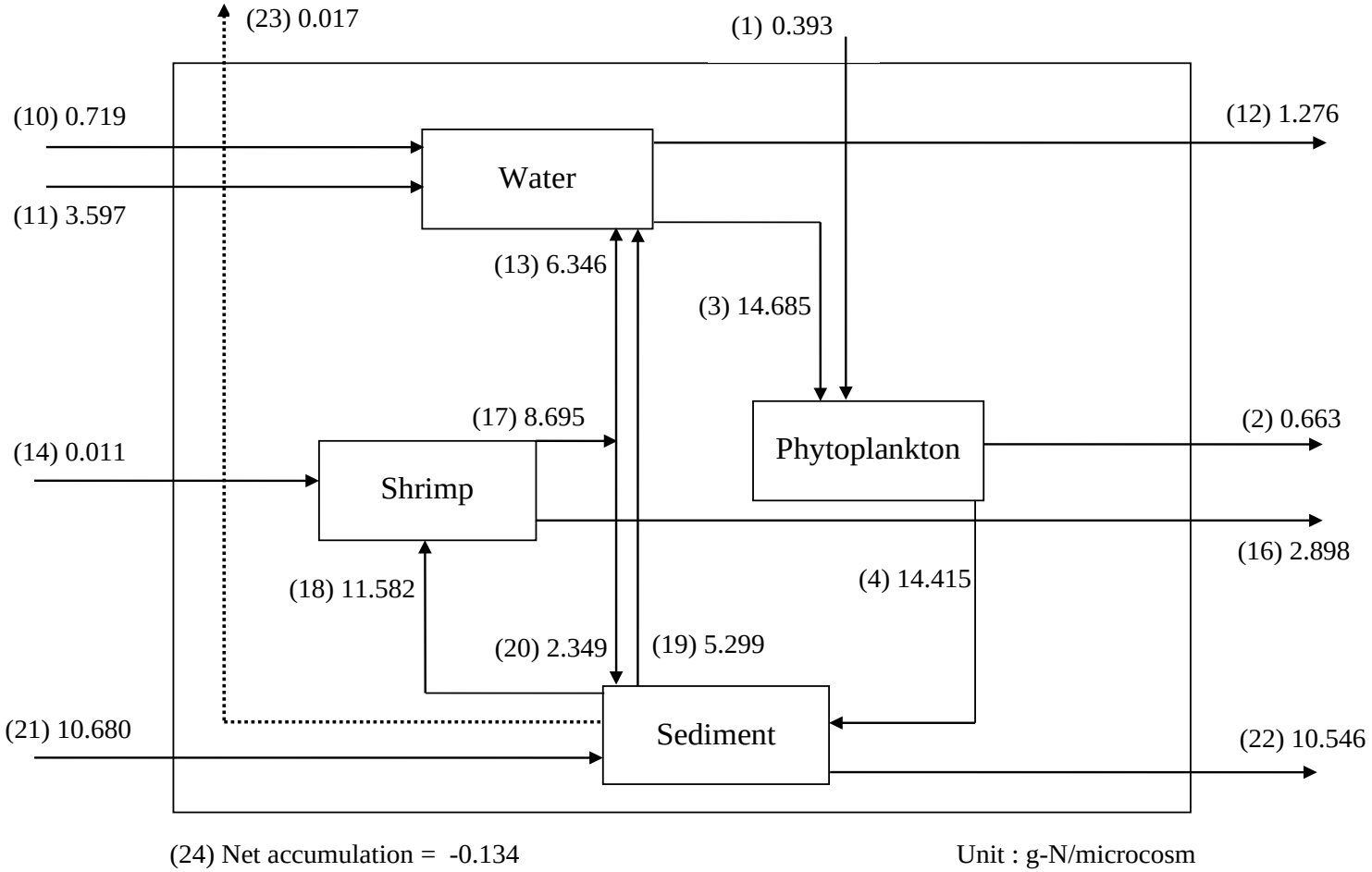

Fig. 6-2 Model of the transformation of nitrogen in white shrimp microcosm without pellet feeding (T1, control) consisting of 4 subunits, namely water, phytoplankton, shrimp and sediment.

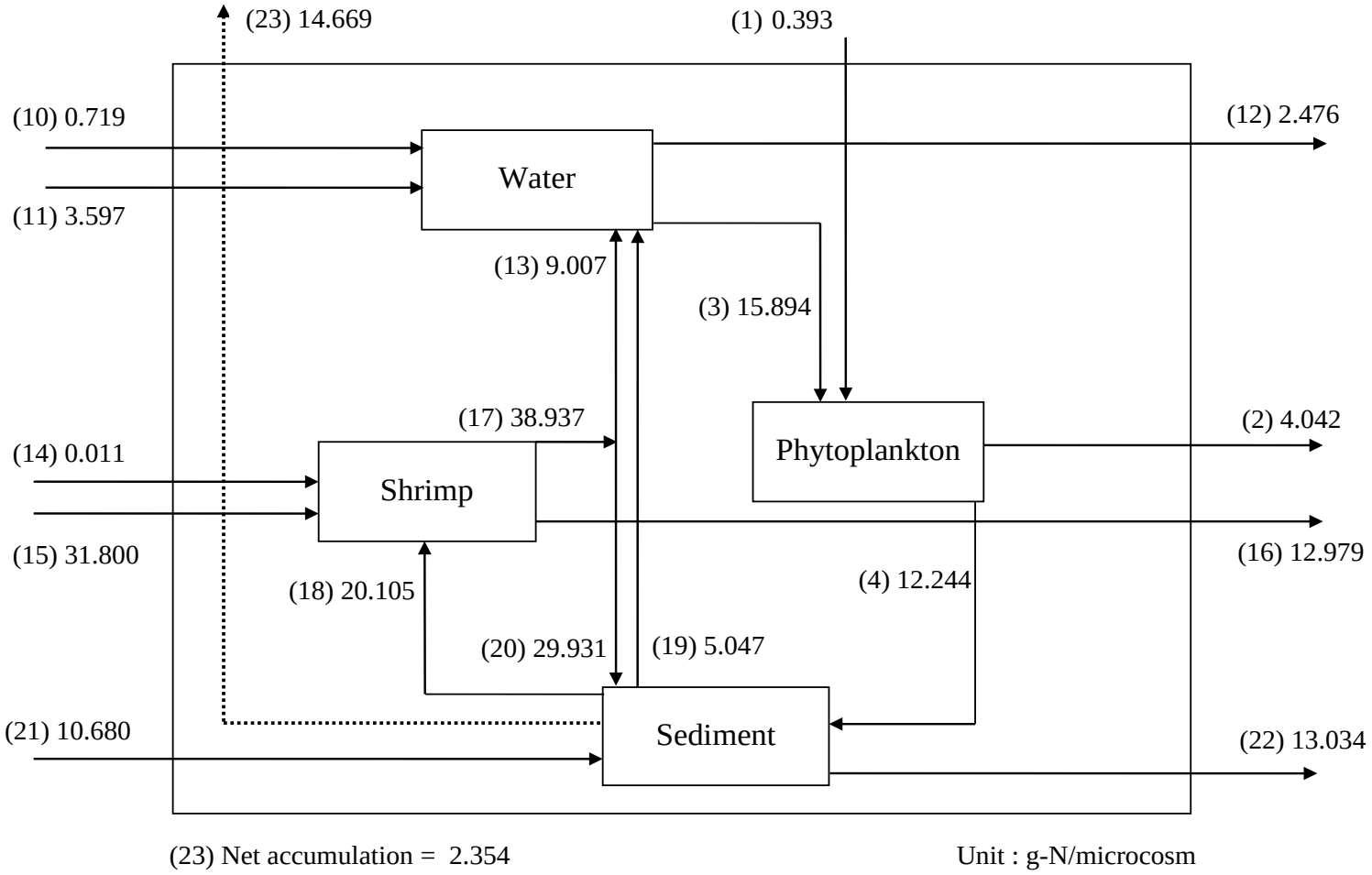

Fig. 6-3 Model of the transformation of nitrogen in white shrimp microcosm with pellet feeding (T2) consisting of 4 subunits, namely water, phytoplankton, shrimp and sediment.

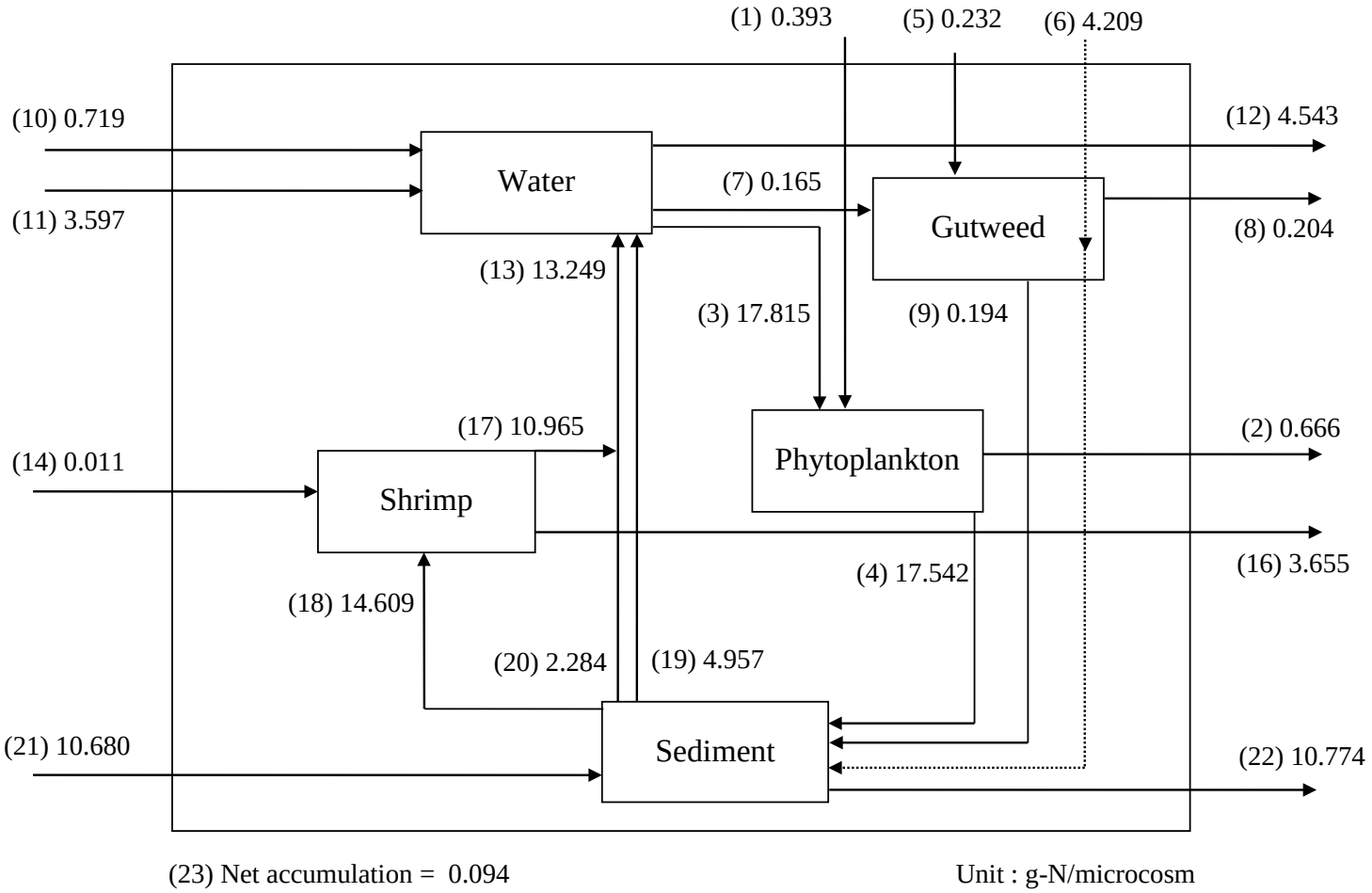

Fig. 6-4 Model of the transformation of nitrogen in white shrimp microcosm without pellet feeding but with gutweed planted (T3), consisting of 5 subunits, namely water, phytoplankton, gutweed, shrimp and sediment.

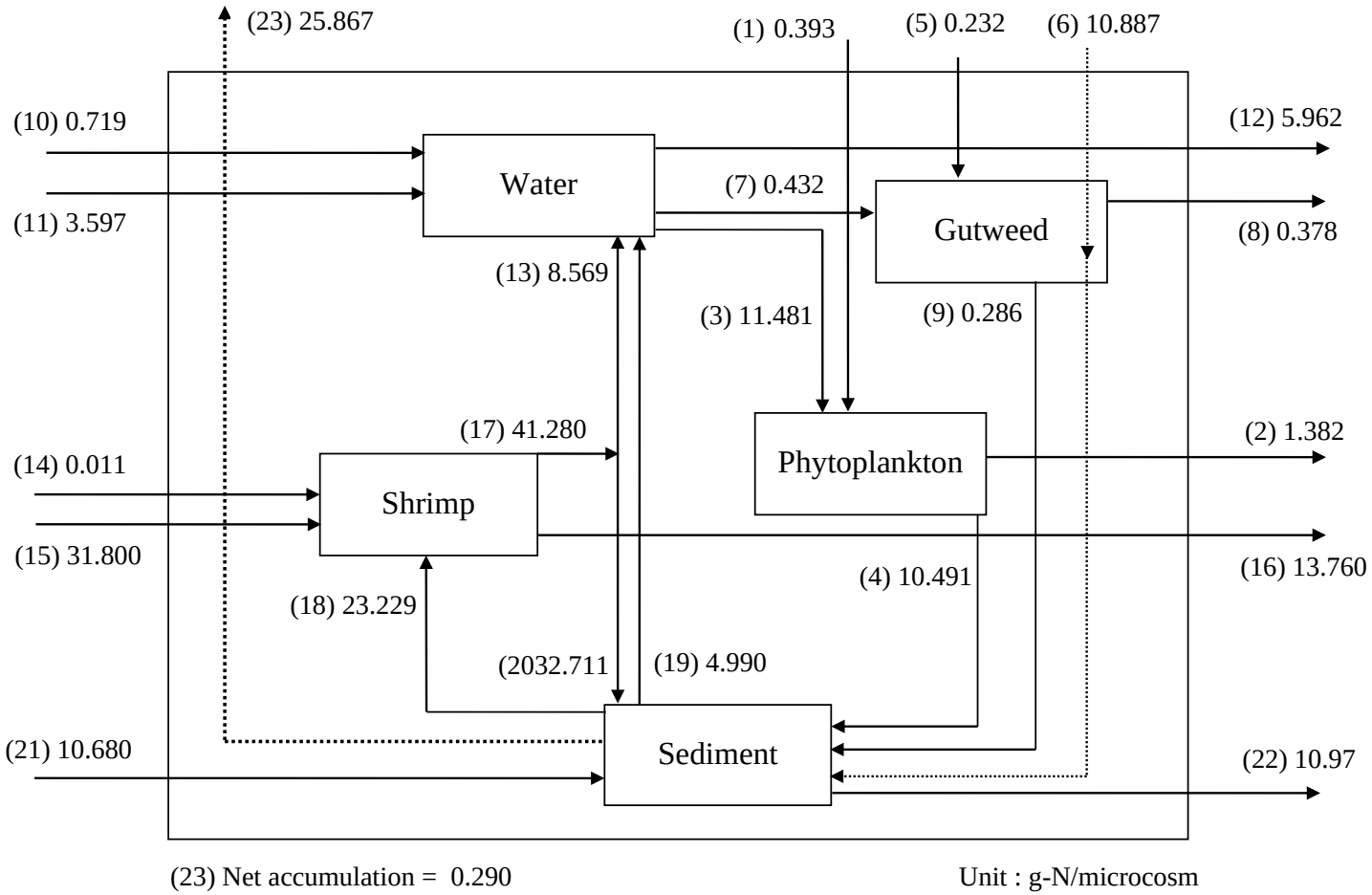

Fig. 6-5 Model of the transformation of nitrogen in white shrimp microcosm with pellet feeding and gutweed planted (T4), consisting of 5 subunits, namely water, phytoplankton, gutweed, shrimp and sediment.

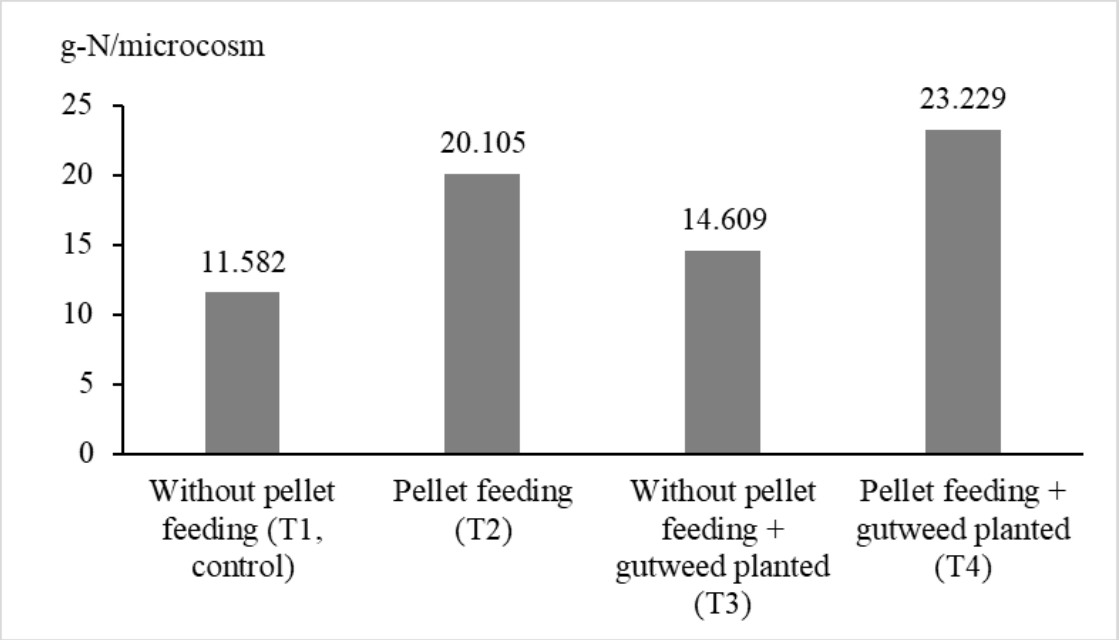

Fig. 6-6 The nitrogen of insect larvae + productivity in white shrimp microcosms cultured for 5 weeks with different feeding regimes [without pellet feeding and with no gutweed planted (T1, control), with pellet feeding (T2), with gutweed planted (T3) and with pellet feeding and gutweed planted (T4)].

Table 6-1 Survival rate and biomass of white shrimps cultured for 5 weeks in different feeding regimes [without pellet feeding and no gutweed planted (T1, control), with pellet feeding (T2), with gutweed planted (T3) and with pellet feeding and gutweed planted (T4)]

Treatment	**Survival rate (%)**	**Biomass (g)**
Control (T1)	80.0 ± 18.1[a]	111 ± 20.3 [a]
Pellets (T2)	91.2 ± 2.26[a]	502 ± 8.20 [b]
Gutweed (T3)	86.0 ± 8.49 [a]	141 ± 4.44 [a]
Gutweed + Pellets (T4)	98.0 ± 1.70 [a]	554 ± 17.9 [c]

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSION

The rapidly growing shrimp aquaculture is very important to the socio-economic development in southeastern Asian countries, including Thailand. Since 2009, intensive and super-intensive farms have become dominant. Without treatment or management, however, shrimp farming with high stocking density releases into the natural environment much waste water and sludge, which are the main causes of water source pollution (eutrophication) and deterioration. (Chua et al., 1992; Phillips et al., 1991; Beveridge et al., 1994; Wu et al., 1994; Hargreaves, 1998; Naylor et al., 1998).

The new aquaculture technology focuses on reducing bad effects of waste and supporting environment-friendly shrimp aquaculture. This also reduces the use of protein sources such as fish meal from the ocean as ingredients for aquafeeds.

Insects have been highlighted by the Food and Agricultural Organization of the United Nations as a sustainable high protein feed ingredient (FAO, 2013). Generally, depending on the species, stage of development (larval, pupae, nymph, adult) and diet, insects can be highly nutritious and are a good source of proteins, lipids, minerals, vitamins and energy (Barroso et al., 2014; Makkar et al., 2014; Nowak et al., 2016).

There is increasing interest in edible insects as an alternative protein source for animal feed as well as for human consumption (van Huis et al., 2013). The insect species *Tenebrio molitor*, also known as the mealworm in the larval stage, is a coleopteran widely cultured for use in pet feed (Finke and Oonincx, 2014) and it has already been tested in diets to replace fishmeal in fish and shrimp farming (Roncarati et al., 2015; Gasco et al.,

2016; Panini et al., 2017a,b: Piccolo et al., 2017). Other species of insect larvae, particularly chironomid (midge) and mosquito larvae that occur naturally in shrimp ponds, may serve as natural food for shrimps. Still, however, there are few reports on the possibility and potential of using naturally-occurring insect larvae as protein sources. This study aims to evaluate the potential of chironomid and mosquito larvae, as a protein source that can replace pellet feed. The studies have been extended to stimulating the occurrence of insect larvae in shrimp ponds by planting the gutweed (green alga: *Ulva intestinalis*) and raking sludge so that this can serve as a source of nutrients.

The objective of this study was to investigate the occurrence and levels of nitrogen in shrimp-culture microcosms with different feeding regimes and the effect of gutweed on their nitrogen budget, the importance of naturally-occurring insect larvae and gutweed as protein sources and as complementary food in shrimp aquaculture, and finally the use of gutweed and raking pond sludge to enhance the abundance of insect larvae. Overall, the study aims to establish an improved practice for shrimp aquaculture, that has both economic and environmental benefit. The experimental work is described in five chapters.

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Chapter II. Comparison of the occurrence and levels of different forms of nitrogen in shrimp culture microcosms with different feeding regimes.

This evaluation of the occurrence and levels of different forms of nitrogen in shrimp culture microcosms with different feeding regimes showed that there was a high fluctuation of nitrogen compounds in pellet-feeding microcosms with and without gutweed planted (T2 and T4), especially of harmful forms of ammonia nitrogen (Fig.2-1A) and nitrite (Fig. 2-1B). Yang et al. (2017) reported that high ammonia concentrations

in a shrimp-pond water column might be attributed to the high rates of ammonia release from the shrimp pond sediment. In the present study, the concentration of nitrogen in water and sediment was higher in the pellet-feeding microcosm (T2).

Organic nitrogen was also accumulated in the sediment. These results indicate that nitrogen from feed was accumulated at a high level in intensive shrimp ponds. Many studies have been published about this. Lin and Nash (1996) reported that 26% of the nitrogen applied as feed accumulated in the sediments of intensive shrimp ponds, whereas Funge-Smith and Brigges (1998) reported a value of 24% in the sediment of a shrimp pond in Thailand. Planting gutweed could reduce the accumulation of nitrogen in water and sediment of shrimp ponds, resulting in a lower nitrogen release into the water column.

Chapter III. Evaluation of the importance of naturally-occurring insect larvae and gutweed as complementary food for white shrimp aquaculture.

The investigation of the feeding behaviour and the digestion of insect larvae showed that juvenile white shrimps *Litopenaeus vannamei* consumed both animal and plant matter, but preferred animal-derived to plant-derived food. Chironomid larvae were the most acceptable food; nearly 100% of chironomid larvae were consumed within 6 hrs (Fig.3-2). The results given in Fig. 3-3 show that shrimps needed only a short time to digest chironomid larvae, so shrimps fed with chironomid larvae revealed the best growth (Fig. 3-4) and had the highest FCE (food conversion efficiency) (Fig. 3-5). No adverse effects on health were seen. Mosquito larvae have a refractory cuticle of chitin, a polymer of *N*-acetyl-β-D-glucosamine (Merzendorfer and Zimoch, 2003) whilst chironomid larvae are worm-like and have soft skin. Gutweed was also eaten and would be a good source of nutrients but was digested only slowly, so it is not such a good food source as the insect larvae

Chapter IV. Evaluation of these natural foods as protein sources and for partial replacement of pellet feed.

Chironomid larvae have a high protein content of 62%, similar to that of fishmeal (60-72%) (Cho and Kim, 2011), as do mosquito larvae. The protein content and the concentrations of EAA (essential amino acids) and NEAA (non-essential amino acids) in the chironomid and mosquito larvae were higher than in gutweed. (Table 4-1). Plant products usually have a lower content of protein and amino acids than animal products do (Hardy, 2010; Tantikitti, 2014). The protein content of larvae of chironomid and mosquito was higher than in most insect adults and larvae previously analysed, such as the adult variegated grasshopper, black soldier fly larvae, housefly maggot meal or silkworm pupae meal (Alegbeleye et al., 2012; Tran et al., 2015). The NPU is directly related to shrimp growth. Shrimps were able to convert protein from the chironomid larva into that in the shrimp body at a higher level (36% of the protein) than from the other natural foods and at a similar level to that from pellets (Table 4-3).

Pellets are produced to be a rich source of protein, and contain other nutrients, such as lipid, vitamins and minerals and essential fatty acids, suitable for shrimp growth (McLamey et al., 1974; Habib et al., 1997). The larvae also have high contents of essential amino acids, (Habib et al., 1997; Habashy, 2005). Evaluation of the effect of feeding combinations of foods revealed that shrimp fed on the combination of chironomid larvae and pellets (50% or 75% of insect larvae + 50% or 25% of pellets) can be more effective than feeding pellets or insect larvae alone (Table 4-4). The combination of chironomid larvae and pellets was particularly effective. Up to 75% of pellet feed could be replaced by the insect larvae (chironomid or mosquito larvae) without any negative effect on shrimp growth, thus reducing the cost of providing feed for shrimp aquaculture.

Chapter V. Investigation of the enhancement of the abundance of insect larvae by gutweed and raking of pond sludge.

The investigation of the role of gutweed (*Ulva intestinalis*) to stimulate the occurrence of insect larvae to support the growth of juvenile shrimps showed that the number of insect larvae was related to the biomass of gutweed planted. Chironimid larvae were more highly associated with gutweed than were mosquito larvae. Chironomid larvae are benthic organisms; the larvae will settle and use small pieces of debris and fine sand to build nest-tubes on the surface or in crevices of dead coral skeleton or other substrates (Soong et al., 1999). The study showed that after 2 weeks of shrimp culturing, numbers of chironomid larvae decreased substantially to nearly zero in all treatments (Fig. 5-5a), whilst the number of chironimid larvae in the microcosms without shrimps still increased in all treatments (Fig. 5-1a).

These results show that the chironomid larvae were consumed by shrimps. The stimulation of the abundance of insect larvae by raking sludge and planting gutweed showed that shrimps could grow for 4 weeks without any pellet food in the gutweed-planted tanks (T6) (Fig. 5-4). So gutweed may play an important role as substratum or shelter to which chironomid larvae could attach and settle into the thallus where they can find plant debris and particulate matter. Raking pond sludge stimulated the occurrence of sufficient insect larvae for 3 weeks of shrimp growth. It is concluded that gutweed stimulated the occurrence of insect larvae, probably by providing important shelter for chironomid larvae.

Chapter VI. Investigation of the role of gutweed in the nitrogen budget of shrimp microcosms with different feeding regimes.

A static model was used for calculation of nitrogen budgets in shrimp ponds with different feeding regimes, to show the role of gutweed on transformation of nitrogen from sediment through naturally-occurring insect larvae to shrimp biomass. Raking the sediment released nutrient (nitrogen) to the water subunit, and this was transferred to primary productivity (phytoplankton and gutweed) by assimilation. Insect larvae contributed to this pathway in the shrimp pond with planted gutweed and their contribution was positively related to the gutweed biomass.

Shrimps fed on pellets in the microcosm with planted gutweed (T4) had the highest natural productivity from insect larvae (Figs. 6-5, 6-6). Furthermore, this gave the highest survival rate and higher shrimp biomass (Table 6-1) when compared to microcosms in which shrimps were fed pellets but no gutweed was planted

These results were consistent with the role of gutweed as a shelter in shrimp ponds (Muangyao et al., 2019c). The pond with pellet feeding had the highest nitrogen accumulation in sediment, thus giving poor quality of environment that could poison shrimps living in the pond. The results also showed that when gutweed was planted, however, the concentration of nitrogen in water and sediment was eight times lower than in the microcosm in which pellets were fed but no gutweed was planted (Figs. 6-3 and 6-5).

When taken together, the results reported in this dissertation confirm the importance of insect larvae, especially chironomid larvae, for the growth of shrimps and also the roles of gutweed in stimulating the abundance of insect larvae. During preparation of a shrimp pond to use organic matter as a nutrient source for insect larvae, putting only a little water into the pond at first, to give a water depth of 15-30 cm, and

raking the bottom sediment to resuspend a high concentration of organic matter will enhance the abundance of insect larvae. This treatment would also reduce the impact of waste from shrimp culture on the environment. Enhancing the population of insect larvae in a shrimp pond, therefore, can reduce the amount of expensive protein-rich feed that needs to be supplied, thereby reducing the cost of producing cultured shrimps.

SUMMARY

Shrimp aquaculture is very important for socioeconomic development in southeastern Asian countries. Shrimp farming with high stocking density releases much waste water and sludge, which often results in water pollution (eutrophication) and deterioration., Several reports have pointed out the importance of benthic insect larvae for decreasing the organic waste in shrimp-pond sediment in shrimp aquaculture. This study has evaluated the potential of gutweed and of chironomid and mosquito larvae, as protein sources that can replace pellet feed. The studies have been extended to show that the occurrence of insect larvae in shrimp ponds can be enhanced by planting gutweed and using sludge as nutrient source. As the results of these studies show, new information was obtained, as summarized below.

1. Planting gutweed in a pellet-feeding shrimp pond could reduce the accumulation of nitrogen in sediment and also reduce the concentration of nitrogen in the water column.
2. Naturally-occurring chironomid and mosquito larvae, besides the green alga gutweed, were tested as food for juvenile white shrimps. Chironomid larvae were highly acceptable and the most effective food.

3. White shrimps were able to convert protein from the chironomid larva into that in the shrimp body at a higher level than from the mosquito larvae or gutweed and a similar level to that from pellets.
4. White shrimps fed on chironomid larvae had high growth, comparable to that of shrimps fed on pellets, so chironomid larvae may be used as a substitute for expensive protein-rich feed for shrimps.
5. Feeding a combination of pellets and insect larvae can be more effective than feeding pellets or insect larvae alone and feeding on 50% of chironomid larvae and 50% of pellets was particularly effective.
6. Up to 75% of pellets could be replaced by chironomid or mosquito larvae, thus reducing the cost of providing feed for shrimp aquaculture.
7. The number of insect larvae is related to the biomass of gutweed planted in a shrimp pond. Chironomid larvae were more highly associated with gutweed than were mosquito larvae.
8. Simply raking the sediment in order to release organic matter stimulates the occurrence of enough insect larvae to support juvenile shrimp growth for up to 3 weeks, but when gutweed is planted, the increased abundance of insect larvae supports growth for at least 4 weeks.
9. Planting gutweed enhanced the cycling of nutrients from sediment through insect larvae and natural productivity to shrimp biomass.

The results obtained here do raise the question of whether there may be some as yet unidentified factor in the chironomid larvae that stimulates the uptake of protein from pellets, or feeding the combination of pellets and insect larvae may give a more suitable

nutritional balance for juvenile white shrimp. This study investigated only juvenile shrimps; a study on post-juvenile shrimps should be undertaken in the future.

In conclusion, this dissertation reports studies on the use of naturally occurring insect larvae for eco-friendly aquaculture of shrimps and provides a sound basis for the use of gutweed and sludge raking to enhance the amount of natural food in the shrimp pond. The results of this work can be used to recommend an improved practice for shrimp aquaculture that has economic benefit by reducing the need to supply expensive commercial pellet feed, and at the same time minimize eutrophication and the release of pollutants into the environment. This would benefit farmers by reducing production costs. It would also have a major effect for the general population by supporting the sustainable development goals and protecting the natural environment.

APPENDIX

Table 1 Nitrogen transformation in white shrimp microcosms cultured for 5 weeks with different feeding regimes

Subunits	No.	Flow	Without pellet feeding (T1, control)	Pellet feeding (T2)	Without pellet feeding + gutweed planted (T3)	Pellet feeding + gutweed planted (T4)
			g-N/microcosm	g-N/microcosm	g-N/microcosm	g-N/microcosm
Phytoplankton	1	phytoplankto input	0.393	0.393	0.393	0.393
	2	phytoplankto output	0.666	4.042	0.666	1.382
	3	phytoplankto assimilation	14.685	15.894	17.815	11.481
	4	phytoplankto sink	14.415	12.244	17.542	10.491
Gutweed	5	gutweed input			0.232	0.232
	6	insect larvae contribution			4.209	10.887
	7	gutweed assimilation			0.165	0.432
	8	gutweed output			0.204	0.378
	9	gutweed sink			4.403	11.173
Water	10	water input	0.719	0.719	0.719	0.719
	11	water addition	3.597	3.597	3.597	3.597
	12	water output	1.276	2.476	4.543	5.962
	13	excretion + solubilizing	6.346	9.007	13.249	8.569
	19	sediment raking	5.299	5.047	4.957	4.990
Shrimp	14	shrimp input	0.011	0.011	0.011	0.011
	15	feed input		31.803		31.800
	16	shrimp output	2.898	12.979	3.655	13.760
	17	shrimp excretion	8.695	38.937	10.965	41.280
	18	insect larvae + natural productivity	11.582	20.105	14.609	23.229
Sediment	19	sediment raking	5.299	5.047	4.957	4.990
	20	sediment solubilizing	2.349	29.931	2.284	32.711
	21	sediment input	10.680	10.680	10.680	10.680
	22	sediment output	10.546	13.034	10.774	10.970
	23	denitrification	0.017	14.669	0.000	25.867
	24	net accumulation	-0.134	2.354	0.094	0.290

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