Effects of Melamine and Urea formaldehyde contamination in Nile tilapia (*Oreochromis niloticus*) diets on growth performance, economic loss and toxicity appearance characteristic index

Aruneepong Srisathaporn[1], Jowaman Khajarern[1*], Prapas Chalorkpunrut[2] and Bundit Tengjroenkul[3]

บทคัดย่อ: การเสริมเมลามีนร่วมกับยูเรียฟอร์มัลดีไฮด์ชนิดละ 3 ระดับ (0.5, 1.0 และ 2.0%) ในอาหาร ต่อสมรรถนะการผลิตและลักษณะบ่งชี้ความเป็นพิษในปลานิล วางแผนการทดลองแบบสุ่มสมบูรณ์ เปรียบเทียบกับกลุ่มควบคุมที่ไม่เสริม ใช้ปลาคละเพศน้ำหนักเริ่มต้นเฉลี่ย 9.67±0.03 กรัม จำนวน 480 ตัว เลี้ยงเป็นเวลา 6 สัปดาห์ พบว่าไม่มีสหสัมพันธ์ (interaction) ร่วมระหว่างเมลามีนและยูเรียฟอร์มัลดีไฮด์ที่เสริมทั้งเมลามีนและยูเรียฟอร์มัลดีไฮด์ มีผลแบบเส้นตรง ($P<0.05$) ต่อการลดสมรรถนะการเจริญเติบโต ประสิทธิภาพการใช้อาหาร ลดอัตราการรอดตายต่ำกว่ากลุ่มควบคุม ($P<0.05$) และเพิ่มความสูญเสียทางเศรษฐกิจ (ELI) สูงกว่ากลุ่มควบคุม ($P<0.05$) โดยประเมินจากค่าดัชนีการผลิต (PI) ที่มีค่าลดต่ำลงแบบเส้นตรง ($P<0.05$) ตามระดับการเพิ่มของสารในอาหาร พบความผิดปกติของเกล็ดและสีของเกล็ด พบรอยโรคในอวัยวะภายใน เช่น ตับ ไต ม้าม และรังไข่ มีความเสียหายในระดับรุนแรง ตับและไต มีเลือดออก บวม ขยายใหญ่ มีลักษณะเปราะ ตับซีดไม่มีเลือด มีน้ำดีแผ่กระจาย ม้ามบวมโต มีสีผิดปกติ การตรวจสอบเนื้อเยื่อสดและทางจุลพยาธิวิทยา พบเนื้อเยื่อและโครงสร้างของเซลล์ถูกทำลายเสียหายอย่างรุนแรง พบผลึกสีน้ำตาลและผลึกแวววาวสะท้อนแสงขนาดต่างๆ กระจายทั่วบริเวณที่เสียหาย ซึ่งขนาดและจำนวนของผลึกที่พบสัมพันธ์กับปริมาณของสารที่เสริมในอาหาร จากข้อมูลหลักฐานดังกล่าว สามารถใช้ในเป็นตัวบ่งชี้และทำนายการปนเปื้อนเมลามีนและยูเรียฟอร์มัลดีไฮด์ในอาหารได้

คำสำคัญ: เมลามีน, ยูเรียฟอร์มัลดีไฮด์, กล้องจุลทรรศน์, จุลพยาธิวิทยา, ดัชนีผลผลิต

ABSTRACT: A study was performed to evaluate the effects of melamine (Me) and urea formaldehyde (UF) combinations in diets on growth performance, and characterization of toxicity appearance. The combinations of three levels of Me and UF (0.5, 1.0 and 2.0%) were conducted with one control (no addition) in a completely randomized design (CRD). Four hundred and eighty mixed-sex fish with the initial weight of 9.67±0.03 g were assigned to 10 dietary treatments with 3 replications and fed for 6 weeks. No interaction was observed between Me and UF in this study. However, significant differences ($P<0.05$) in growth performance, feed efficiency and survival rate (SR) between the control and the fish fed with Me and UF combinations were observed. The body weight (BW) and body weight gain (BWG) linearly decreased while protein efficiency ratio (PER), feed conversion efficiency (FCE) and FCR were less efficient linearly ($P<0.05$) with the increasing levels of Me and UF combination, and were less efficient than the control ($P<0.05$). The SR and ELI as determined by PI showed significant linear decreases ($P<0.05$). A linear chronic pattern of appearance characteristic index of scale erosion, lesion and degeneration of viscera organs (liver, kidneys, spleen and ovary) was observed. Microscopic examinations of wet mount tissue and of histological changes of those tissue organs found them to be degenerated and damaged with numerous golden brown or transparent crystals. The crystals found provided evidence of strong correlation between the amounts and sizes of crystals, and the levels of Me and UF in diets. The results of this study indicated that the combinations of Me and UF in diets decreased growth performance, but increased economic loss index (ELI) and appearance characteristic of scale erosion and color change. The degeneration of tissue organs and crystals provided strong evidence in the prediction of Me and UF contamination in diets.

Keywords: melamine, urea formaldehyde, microscope, histology, production index

[1] Department of Animal Science, Faculty of Agriculture, Khon Kean University, Khon Kaen 40002, Thailand

[2] Department of Fishery of Agriculture, Khon Kean University, Khon Kaen 40002, Thailand

[3] Faculty of Veterinary Medicine, Khon Kean University, Khon Kaen 40002, Thailand

* Corresponding author: jowaman@kku.ac.th

Introduction

Melamine (Me), a synthetic chemical (1, 3, 5- triazine -2, 4, 6-triamine) containing 66.6% nitrogen, and widely used in various industries except in animal feed. Me is not approved by the U.S. Food and Drug Administration. In 2007, pet food, wheat flour and animal protein feedstuffs were reported to be contaminated with Me (Anderson et al., 2008). Me has also been found in binding agents for increasing the efficiency of pelleting feed in swine, poultry, livestock and aquatic feed (US-FDA, 2007). Urea formaldehyde (UF) is a synthetic binder that is widely used in aquatic feed to improve the efficiency of feed by reducing water stability for a more water-resistant diet (Muylder et al., 2008). UF has no nutritional value although it contains high nitrogen. Aqua-bond, the commercial brand name of UF, is used as a binder for shrimp feed within the recommended dosage (0.5-1.0%) which has no toxicity and does not modify taste or color of feed.

Protein is an essential nutrient, and the price of a protein source depends on the protein content. These lead to the addition of either Me or UF in feedstuffs, such as fish meal or fish (Karunasagar, 2008). Therefore, it is important to monitor both animal feedstuffs and animal tissue for Me and UF contamination. However, there are relatively few methods for the determination of Me or UF in animal feed and animal tissue (Anderson et al., 2008). Due to the fact that Me was considered to be relatively nontoxic (Melnick et al., 1984), the data on the study of the effects of Me or UF in animals was lacking in the past. For instance, in Thailand, Janlek et al. (2009), Kateprakob and Phromkunthong (2011), and Nuntapong and Phromkunthong (2011) had showed results on low growth performance and some physiological damage. Therefore, this study was conducted with the Nile tilapia to evaluate the effects of Me and UF combinations on growth performance , and appearance characteristic lesions as the indicators for evaluating the significant effects of contamination in fish diets.

Materials and Methods

The combinations of Me and UF at 0.5, 1.0 and 2.0% was composed with one control (no addition) in a completely randomized design (CRD). The basal diet was formulated to contain 32% protein **(Table 1,2)**. The 10 contamination treatments with one control diet were prepared. The 480 mixed-sex Nile tilapia fingerlings with the average initial weight of 9.67±0.03 grams were randomly assigned to 30 glass aquaria (30x40x30 cm^3) with constant aeration. The 10 experimental diets were randomly assigned in 3 replicates. They were fed twice daily at 9.00 am and 15.30 pm. Refused feed and feces were siphoned off every morning, and water was replaced before feeding.

The fish of all replicates were weighed and feed intake was recorded every two weeks. Mortality was recorded daily during the period of experiment. At the end of the 6^{th} week, 9 fish from each treatment were sampled and anesthetized in cold water. A part of the sampled organs were fixed in 10% buffered neutral formalin for histological examination. The remaining samples were kept at -20°C for microscopic characterization examination.

Measurements and statistical analysis

All data were evaluated for body weight (BW), body weight gain (BWG), percentage of weight gain (%WG; = weight gain x 100/initial weight), specific growth rate (SGR, %/d; = (ln final weight - ln initial weight) x 100/days), feed conversion ratio (FCR), protein efficiency ratio (PER, %; = weight gain (g) x 100/protein intake (g)), feed conversion efficiency (FCE; = WG x 100/feed intake), and survival rate (SR, %; = no. of remaining fish x 100/initial no. of fish). The economic loss index (ELI) was evaluated at the end of the experiment by using the production index (PI) which was calculated by weight gain x survival rate (%)/FCR. At the end of the experiment, the fish were recorded for the gross lesions (diffuse hemorrhage, enlargement, necrosis and color change) of liver, kidneys, spleen and reproductive organs under a light microscope (Motic SFC-11 with Sony color video camera, model SSC-E458P).

All treatments data were analyzed using one-way analysis of variance (ANOVA) tests, and means were compared by Duncan's multiple range tests at $P<0.05$ (Steel and Torrie, 1980).

Table 1 Composition of the basal diet (as-fed basis) used in the control treatment.

Ingredient	Percentage %
Fish meal CP 64 %	26.0
Soybean meal CP 46%	17.7
Full fat soybean CP 36%	10.0
Cassava meal	10.0
Corn meal	10.0
Rice bran	19.0
Wheat flour	4.0
Dicalcium phosphate, P18	1.0
DL-Methionine 98 %	0.2
L-Lysine 98 %	0.1
Vitamin-mineral premix[1]	2.0
Total	100.0
Calculations:	
CP, %	32.2
GE, kcal/kg[2]	4,258.0
ME, kcal/kg	3,001.0

[1]Supplied per kilogram of diet:

Premixed vitamins: Vitamin A, 30,000 IU; D3, 6,000 IU; E, 90 mg; K3, 6 mg; B1, 8 mg; B2, 10 mg; B12, 0.02 mg; Pantothenic acid, 30 mg; Nicotinic acid, 60 mg; Folic acid, 3 mg; Biotin, 0.6 mg; Vitamin C, 150 mg; Preservative, 2 mg; Anti-caking agent, 10 mg; Carrier, added up to 1000 mg; Choline Chloride 50% N, 1 g

Premixed minerals: Mn, 20 mg; Zn, 10.5 mg; Cu, 4 mg; Co, 5 mg; Se, 0.3 mg; Fe, 75 mg; I, 3 mg; Mg, 90 mg; Anti-caking agent, 10 mg; Carrier, added up to 1000 mg

[2] GE calculation from (CP x 5.64 kcal/g) + (EE x 9.44 kcal/g) + (CHO x 4.063 kcal/g)

Table 2 Experimental diets, chemical composition of the combinations of Me and UF by calculation, and proximate analysis.

	Experimental Diet									
Composition, %	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
Basal diet*	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Me	0.0	0.5	0.5	0.5	1.0	1.0	1.0	2.0	2.0	2.0
UF	0.0	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0
Total	100.0	101.0	101.5	102.5	101.5	102.0	103.0	102.5	103.0	104.0
Crude protein (CP) by calculation (%):										
CP from basal feed	32.2	32.2	32.2	32.2	32.2	32.2	32.2	32.2	32.2	32.2
CP from Me	0.0	2.1	2.1	2.1	4.2	4.2	4.2	8.4	8.4	8.4
CP from UF	0.0	1.1	2.2	4.4	1.1	2.2	4.4	1.1	2.2	4.4
CP in total diet	32.2	35.4	36.5	38.7	37.5	38.6	40.8	41.7	42.8	44.8
CP/100kg	32.2	35.1	36.0	37.8	36.9	37.8	39.6	40.7	41.6	43.1
CP by proximate analysis (%): 32.2		33.6	35.3	35.5	37.3	37.5	38.2	37.1	38.5	39.3

Results

Growth performance and feed efficiency parameters

The data showed no significant effect of interaction (P>0.05) between Me and UF in diets **(Table 3)**. Fish of all groups fed with the combinations of Me and UF showed significant effects (P<0.05) on growth when compared to the control, i.e., lower growth rate. Me showed highly significant linearly decreasing (P<0.01) effects on BW, BWG, %WG and SGR; while RGI showed tendency of a linearly decreasing (P=0.06) effect when Me levels increase. UF showed a significant linearly decreasing (P<0.05) effect on growth in terms of SGR.

The fish of all groups fed with Me and UF combination diets showed significant effects (P<0.05) on feed efficiency, SR and PI **(Table 4)** by having less efficient FCR, lower PER and FCE (P<0.01), and also lower SR and PI than the control group. Me showed no significant effect (P>0.05) on SR but showed a significant effect on PI, while UF showed highly significant linear

effects (P<0.01). Me showed more significant depressing (P<0.05) effects on growth than UF. The depression was related to the increase of Me in diets. However, both Me and UF showed significant linear effects (P<0.05) of reduced feed efficiency as their levels increased in diets. There was also a significant linearly increasing (P<0.05) effect on SR as the levels of UF in diets increased (Table 5).

Productive index (PI) showed greatly significant decreases (P<0.05) in all groups fed with Me and UF combination diets when compared with the control. Both Me and UF affected ELI by showing significant linearly decreasing effects (P<0.05) on PI: PI decreased as Me and UF levels increased in diets, and the greatest decrease was shown at the highest Me and UF level in diets.

Table 3 Effects of melamine (Me) and urea formaldehyde (UF) combinations in diets on growth performance parameters of tilapia after feeding for 6 weeks.

Treatment diet, %		Growth performance parameter				
Me	UF	BW, g	WG, g	%WG	SGR,%/d	RGI,%
0.0	0.0	18.19^{a}	14.63^{a}	411.23^{a}	3.62^{a}	100.00
0.5	0.5	17.44^{ab}	13.84^{ab}	384.30^{ab}	3.50^{ab}	94.68
0.5	1.0	17.24^{abc}	13.54^{abc}	364.81^{abc}	3.43^{abc}	92.62
0.5	2.0	16.76^{abc}	13.15^{abc}	363.76^{abc}	3.45^{abc}	89.92
1.0	0.5	16.90^{abc}	13.31^{abc}	367.68^{abc}	3.45^{abc}	91.07
1.0	1.0	16.58^{abc}	13.05^{abc}	369.07^{abc}	3.452^{bc}	89.31
1.0	2.0	15.76^{bc}	12.12^{bc}	328.01^{c}	3.26^{cd}	82.92
2.0	0.5	16.18^{bc}	12.59^{bc}	350.03^{bc}	3.67^{bc}	86.04
2.0	1.0	15.81^{bc}	12.20^{bc}	335.69^{bc}	3.34b^{cd}	83.57
2.0	2.0	15.60^{c}	11.93^{c}	324.67c	3.16^{d}	81.40
SEM		0.51	0.52	15.69	0.06	4.13
P-value		*0.034*	*0.031*	*0.024*	*0.002*	0.097
Control vs others	*0.005*		*0.004*	*0.003*	*0.001*	*0.012*
Me		*0.021*	*0.024*	*0.048*	*0.008*	0.060
UF		0.181	0.155	0.105	*0.017*	0.240
Me (lin)		*0.006*	*0.007*	*0.015*	*0.002*	*0.020*
Me (qua)		0.802	0.899	0.926	0.938	0.930
UF (lin)		0.071	0.059	*0.038*	*0.006*	0.100
UF (qua)		0.776	0.770	0.7659	0.463	0.780
Me x UF		0.967	0.994	0.951	0.230	0.780
Me x UF (lin)		0.576	0.578	0.546	0.602	0.640
Me x UF (qua)		0.720	0.772	0.666	0.270	0.603

^{abcd} Value within the column with a common superscript are significantly different (P<0.05)

Relative growth index (RGI, %) = (final weight - initial weight) x 100/control weight

Table 4 Effects of melamine (Me) and urea formaldehyde (UF) combinations in diets on feed efficiency parameters of tilapia after feeding for 6 weeks.

Treatment diet, %		Feed efficiency parameter				
Me	UF	FCR	PER,%	FCE,%	SR,%	PI
0.0	0.0	1.14^{d}	2.73^{a}	88.06^{a}	97.92^{a}	1262.3^{a}
0.5	0.5	1.17^{cd}	2.58^{ab}	85.56^{ab}	91.67^{ab}	1086.5^{ab}
0.5	1.0	1.24^{bcd}	2.37^{bc}	81.03^{abc}	85.42^{abc}	935.9^{bc}
0.5	2.0	1.32^{abc}	2.10^{de}	75.88^{bcd}	72.92^{cd}	736.7^{cd}
1.0	0.5	1.23^{bcd}	2.43^{bc}	81.65^{abc}	89.58^{abc}	970.9^{bc}
1.0	1.0	1.26^{abcd}	2.25^{cd}	79.56^{abcd}	87.50^{abc}	917.3^{bc}
1.0	2.0	1.37^{ab}	2.06^{de}	73.15^{cd}	79.17^{bcd}	705.8^{cd}
2.0	0.5	1.31^{abc}	2.06^{de}	76.71^{bcd}	83.33^{abc}	821.0^{bcd}
2.0	1.0	1.39$_{ab}$	1.93^{e}	72.33^{cd}	81.25^{abc}	734.2^{cd}
2.0	2.0	1.42^{a}	1.85^{e}	70.69^{d}	64.58^{d}	541.5^{d}
SEM		0.05	0.08	2.96	5.06	91.69
P-value		*0.007*	*0.001*	0.006	*0.008*	*0.001*
Control vs others		*0.004*	*0.000*	*0.003*	*0.006*	*0.000*
Me		*0.014*	*0.000*	*0.017*	0.098	*0.021*
UF		*0.002*	*0.000*	*0.012*	*0.002*	*0.002*
Me (lin)		*0.004*	*0.000*	*0.005*	0.108	*0.008*
Me (qua)		0.553	0.107	0.608	0.136	0.403
UF (lin)		*0.003*	*0.000*	*0.003*	*0.001*	*0.001*
UF (qua)		0.822	0.957	0.862	0.221	0.431
Me x UF		0.837	0.266	0.785	0.894	0.914
Me x UF (lin)		0.894	0.873	0.999	0.353	0.758
Me x UF (qua)		0.561	0.957	0.552	0.786	0.772

^{abcd} Value within the column with a common superscript are significantly different (P<0.05)

Table 5 Growth performance and feed efficiency of Nile tilapia fed with diets containing different levels of melamine (Me) and urea formaldehyde (UF) for 6 weeks.

Me	Growth performance parameter					Feed efficiency parameter				
Level, %	BW,g	WG,g	%WG	SGR,%/d	RGI,%	FCR	PER,%	FCE,%	SR,%	PI
0.5	17.14[a]	13.51[a]	370.96	3.46[a]	92.41	1.24[b]	2.35[a]	80.82[a]	83.33	919.72[a]
1.0	16.41[ab]	12.83[ab]	354.92	3.38[ab]	87.77	1.29[ab]	2.25[a]	78.12[ab]	85.42	864.67[a]
2.0	15.87[b]	12.24[b]	336.8	3.29[b]	83.67	1.37[a]	1.94[b]	73.24[b]	76.39	698.93[b]
P-value										
Me	*0.029*	*0.032*	0.058	*0.010*	*0.074*	*0.02*	*0.0001*	*0.023*	***0.119***	*0.029*
UF Level, %	BW,g	WG,g	%WG	SGR,%/d	RGI,%	FCR	PER,%	FCE,%	SR,%	PI
0.5	16.84	13.24	367.34	3.44[a]	90.59	1.24[b]	2.36[a]	81.31[a]	88.19[a]	959.48[a]
1.0	16.54	12.93	356.52	3.40[a]	88.50	1.30[ab]	2.18[b]	77.64[ab]	84.72[a]	862.48[a]
2.0	16.04	12.40	338.81	3.29[b]	84.75	1.37[a]	2.02[c]	73.24[b]	72.22[b]	661.35[b]
P-value										
UF	*0.21*	*0.18*	*0.12*	*0.02*	*0.27*	*0.02*	*0.02*	*0.00*	*0.00*	*0.00*
Me x UF	*0.98*	*0.97*	*0.80*	*0.49*	*0.98*	*0.95*	*0.93*	*0.62*	*0.89*	*0.99*

[abcd] Value within the column with a common superscript are significantly different (P<0.05)

Microscopic examination of tissue organs

Regarding scale erosion and coloration of tilapia fed with the combinations of Me and UF, the darkening and the pale color in some fish were observed. There were high melanophore accumulation and distribution in the scales of the fish fed with Me and UF diets when compared with the control. The gross lesions of liver showed enlargement, diffuse hemorrhage (with the degree of hemorrhage ranging from moderately to severely diffused), more brittle liver, congestion and necrosis. These were also found at a higher degree in the groups fed with either Me or UF at 0.5 to 2.0% of diets **(Figure 1)**. Lesions of kidneys and spleen also observed enlargement, severe hemorrhage, necrosis and nodular kidneys found with the same pattern as the liver. The damage to tissue organs was observed to be more severe with the increasing levels of Me and the increments of UF in diets. The severest lesions were found in the groups fed with the highest level of UF. The lesion appearances of ovary also found damage and degeneration, abnormal shape of oocytes, coagulative necrosis, and liquefaction of the yolk sphere with large vacuole of mature oocytes and irregular wall of oocytes. The severity was more degrees which related to high level of Me and UF in diet **(Figure 2)**.

Wet mount slide and histological changes examination

Most of the obvious changes in the tissue structure were observed in the groups fed with Me and UF in diets, while no change was observed in the tissue of the control group.

Obvious golden brown, pale yellow, or transparent crystals of Me and UF were seen in the kidneys, liver and spleen of the fish fed with Me and UF diets, whilst no crystal was found in the control group. The crystals of Me in the tissue had irregular shapes-from globular to flattened, and from smaller than 2 microns to large enough to be visible to the naked eye. For the crystals of UF, spine radiation was observed. The amounts and sizes of the crystals of Me and UF, and tissue lesions became more abundant and evident with the increasing levels of Me and UF diets.

There was no pathological change observed in the tissue of the fish fed with the control diet while the fish fed with the Me and UF diets showed variations in tissue degeneration. The liver tissue showed hepatocyte swelling, congestion, less prominent cell membrane, red blood cell distension, and sinusoidal dilation. The kidneys showed tubular degeneration, glomerulus degeneration, vascular congestion, and white blood cell infiltration **(Figure 3)**. The spleen tissue showed congestion, white blood cell infiltration, and vacuole distribution. Abundant phagocyte cells were observed in the spleen of the fish fed with high levels of Me and UF combinations. The degree of degeneration and the abundance of phagocyte cells increased with the increasing levels of Me and UF. Histological examination found several sizes of golden brown crystal-like materials of Me and UF in the kidneys, liver and spleen as well as in the interstitial tissue surrounding the renal tubules of the fish fed with the Me and UF diets while fish fed the control diet were not found. The amounts and sizes increased with the increase of Me and UF. In addition, histological lesions were obviously observed in the kidneys, liver and spleen of the fish in all groups fed with Me and UF combinations, from the low levels to the high levels.

Discussion

In the present experiment, the findings revealed that Me and UF did not have any interaction effect on growth and feed efficacy parameters. Therefore, all growth parameters (BW, BWG, %WG and SGR) of the fish showed linear decreases due to Me, while UF only had effects on SGR. The results indicated that Me had more effects on growth than UF. However, both Me and UF linearly showed less efficiency in the feed efficiency parameters (FCR, FCE and PER) of the fish, which was observed in all groups fed with Me and UF combination diets. The present results support the previous studies on feeding Me diets to fish such as Yowarach and Tengjaroenkul (2010) which fed 2.0-16.0% Me diets to tilapia with the initial weight of about 25 g and found that the growth rate decreased, and that the greatest decreases ($P<0.05$) were found in 8.0-16.0% Me diets. Likewise, the study of Nuntapong and Phromkunthong (2011) which fed Me dietaries to sex-reversed red tilapia with the initial weight about 5.63 g and found that the increasing Me levels from 0.5-3.0% in diets resulted in decreased growth and increased FCR. Similar results were also reported by Kateprakob and Phromkunthong (2011) in sea bass with the average initial weight of about 7.5 g which were fed with 0.1-0.8% Me diets for 8 weeks. Feed intake and growth rate decreased in the fish fed with 0.8% Me diet.

The results of these studies mainly concerned Me and concluded that Me in diets from 0.5% decreased growth rate and feed efficiency of fish, and the degree of the decreasing effects were related to the levels of Me in diets. Unfortunately, there had been a lack of data on the feeding of Me and UF diets in fish in the past. In the present study, tilapia with the average initial weight of about 9.67 g were fed with the combinations of graded levels (0.5, 1.0 and 2.0%) of Me and UF in diets. Me and UF showed similar effects on growth and feed efficiency parameters to the previous studies. However, the difference was that the results showed more severity than the other studies. These results revealed that Me and UF contamination in diets was toxic, and the toxicity increased synergistically due to their increasing concentration in diets.

Me and UF in diets showed adverse effects on the quality of diets. Me and UF are known as non-protein nitrogen (NPN) sources. They release nitrogen into diets which the fish are unable to utilize for growth. The evidence showed that FCE and PER decreased as the levels of Me and UF increased in diets. Smith et al. (1994) and Dobson et al. (2008) described that Me could be absorbed in the gastrointestinal tract and sent to the blood vessels, eventually reaching the internal organs. However, the fish cannot utilize it, and thus they need to eliminate it from the body. Usually, the fish eliminate protein, toxins and gas via the gills and feces; however, in the case of excess protein or toxins, the elimination via the normal process becomes a problem. Therefore, the accumulation in body tissue and organs occur, and the fish use energy to eliminate the toxins by increasing feed intake to meet their energy requirements. This increases FCR. The last study by Sirilaophaisan et al. (2010, 2011)—which fed Me, UF and their equal mixture at the levels of 0, 0.5, 0.75 and 1.0% in diets to broilers and layers—found that broilers fed with the 1.0% Me diet and the 0.75% UF diet had depressed growth rate while the equal mixture of Me and UF in diets showed no effect on BWG. In layers, 0.75% Me, UF and their mixture showed decreased growth performance.

The SR of the fish in the present study showed no effects of Me while UF showed linearly decreasing effects ($P<0.05$). The results of Me effects were similar to the reports of Janlek et al. (2009); Yowarach and Tengjaroenkul (2010); Nuntapong and Phromkunthong (2011); and Kateprakob and Phromkunthong (2011). Yowarach and Tengjaroenkul (2010) reported the lethal dose (LD_{50}) of Me at day 60 was 271g/kg of diet (27.1%). This level of Me LD_{50} was found in low toxicity and tilapia had sub-acute or prolong duration to chronic toxic. However, the SR of the present study was affected by UF which showed linearly decreasing effects ($P<0.05$). This might be the effects of free formaldehyde which was slowly released from UF. Maslosh et al. (2005) reported that the significant drawback of UF was the toxicity caused by the presence of free formaldehyde in UF. Javed et al. (2002) reported feeding formalin (37% formaldehyde) at levels of over 10 ml/kg of feed, revealing significant effects on broilers' health and performance. They mentioned that giving UF through feed for prolonged periods affected animal health and performance. However, the different effects of UF on SR were dependent on the animal species and the concentration UF in diets.

Me and UF combinations in the present study caused obvious economic loss. This result was similar to Siriloaphaisan et al. (2011) which fed broilers with Me, UF or their mixture, showing economic loss and quadratic effects as their levels increased in diets. The result was also similar to other toxin contamination in diets as reported by Khajarern et al. (2003) which stated that in ducklings fed with diets contaminated with aflatoxin, the economic loss was high.

Ovary deformation, degeneration and various stages of ovary were also observed in this study. It was observed that in the fish fed with diets with high levels of UF, the various stages of ovary were found. This meant that both Me and UF in diets affected the development of ovaries. The present results agreed with the report of OECD SIDS (1998) that the chronic toxicity of Me in *Salmo gairdneri* and *Jordanella floridae* were in the early stage of age. Similar results on the negative effects on the fish ovary had been reported by Abdelhamid et al. (2010) which said that tilapia fed with dietary supplements of 40 and 60 mg gibberellic acid/kg of diet showed depletion with atresia of oocytes and abnormal shape of oocytes in the vitellogenic yolk stage. The Me, UF and their combinations were reported of their effects on the egg quality of layers by Siriloaphaisan et al. (2011).

The gross lesions of the liver, kidneys and spleen were mostly enlargement, hemorrhage, brittleness, congestion, necrosis, nodular kidneys, and bile diffusion in the liver. The degree of lesions increased in severity in relation to the levels of Me and UF contamination in diets. The results of the present study differed from those reported by Kobayashi et al. (2010) which fed rats with Me and cyanuric acid diets (120 mg/kg/day). The gross appearance of the kidneys was yellow color with small irregularities on the surface; while in the control group (no addition), the color was reddish-brown. In cats, the gross findings atypical of the kidneys were also reported (Puschner et al., 2007).

The wet mount slide examination found crystals in the tissue organs of the fish in groups fed with Me and UF diets. These findings were similar to the report of Dobson et al. (2008) which studied Me and cyanuric acid, and hypothesized that Me and cyanuric acid formation of melamine-cyanurate crystals in the kidneys precipitated in the renal tubules, leading to progressive tubular blockage and degeneration. In this study, it can be assumed that Me and UF might have the same mechanism, being: absorbed in the gastrointestinal tract (GI tract), distributed systemically, and precipitated in the renal tubules; leading to liver, kidney and spleen tissue degeneration. On the other hand, Me and UF may react with one another to form crystals that may impair kidney, liver and spleen function; making the kidney, liver and spleen lesions more severe with the increasing Me and UF contamination in diets. The results confirmed the assumptions of Puchner et al. (2007) and also supported the study of Sirilaophaisan et al. (2011).

Histological examination of tissue mostly found the degeneration of tissue rather than the crystals as compared to the wet mount examination. Crystals were not found in the liver, kidneys and spleen of the control group. This may be because of some crystals being lost in the routine H & E staining for histological preparation (Dobson, et al., 2008). The results agreed with the

previous report of Sirilaophaisan et al. (2011) which found less Me crystals in the liver, kidney and spleen tissue of broilers fed with Me and UF combinations at the levels of 0.75-1.0% in diets, than the detection by wet mount tissue.

Tissue degeneration results were similar to the reports of Yowarach and Tengjaroenkul (2010) and Nuntapong and Phromkunthong (2011) reported in tilapia. Janlek et al. (2009) and Kubkaew et al. (2010) reported in catfish. However, crystals of Me were not reported in these studies except for the study of Kubkaew et al. (2010) which found round green-brown Me crystals deposited in tissue of fish fed with 4.0-16.0%Me diets and Sirilaophaisan et al. (2010; 2011) which found "spoke wheel" appearance in the tissue of liver, kidneys and spleen of broilers fed with 0.5% Me in diets, and crystals in the tissue of laying hens fed with 1.0% Me in diets.

Similar report on the degeneration of liver, kidney and spleen tissue of tilapia had been reported by Deng et al. (2010) and Anh-Tuan et al. (2002) which studied the feeding of aflatoxin B_1 (AFB_1) diets and found hepatic disorder, lipid content decrease, and abnormal hepatic morphology. Anh-Tuan et al. (2002) found lesions only in the liver, reduced hematocrit, histological change of liver, lipofuscin and irregularly-sized hepatocellular nuclei, and severe hepatic necrosis in the fish fed with 100 mg AFB_1/kg of diet. Spleen abnormality, large deposits of haemosiderin and melanin pigments, proliferation of melano-macrophage center (MMC), lymphocytic depletion of the white pulp area (hypocellularity), and presence of vacuoles and necrotic areas were observed in tilapia fed with 75-100% cotton seed meal (CSM) protein replacement (Garcia-Abiado et al., 2004). Although these studies reported similar results in the degeneration or abnormality of liver and spleen, the difference between the present results and these reports is the findings of dark brown or transparent crystals of various sizes—including large white ones visible to the naked eye—in the liver, kidney and spleen tissues. These findings revealed that the dark brown or transparent crystals can be used as an indicator for predicting the contamination of Me or UF in feed.

The severe degeneration of tissue revealed the abnormality of the tissue organs that are related to metabolism, immune response, and detoxification; leading to the abnormal function of these organs. Therefore, the growth rate of the fish and feed efficiency decreased, and the SR also decreased. In general, these results supported the general understanding that these three organs are important in immunological defense mechanism, haematopoiesis, and trapping and clearance of foreign substances from circulation (Garcia-Abiado et al., 2004).

Conclusion

The results indicated that over the 6-week period, Me (0.5-2.0%) in combination with UF (0.5-2.0%) in diets resulted in lower growth than the control. Me and UF also depressed feed efficiency decreasing SR; and showing obvious ELI. The degree of depressing effects of Me and UF was related to the increasing levels of Me and UF in diets. The evidence showed more severe effects of Me on growth than UF at the same levels throughout the experiment. Scale deformation and change in coloration can provide

evidence of unhealthy fish. The lesions of liver, kidneys and spleen with hemorrhage, enlargement, brittleness, nodules and necrosis can also indicate the toxicity of Me and UF in tilapia; with the degree of severity related to their concentration in diets. Ovary deformation and degeneration indicated defects in the reproductive organs of the fish. Tissue organs, kidney lesions, liver and spleen degeneration were found to be correlated to the increments of the levels of Me and UF in diets. The microscopic examination of Me and UF crystals in the liver, kidney and spleen tissues provided evidence of strong correlation between the amounts and sizes of golden brown crystals, of and Me and UF contents in diets. These findings revealed that the dark brown or transparent crystals can be used as an indicator for predicting the contamination of Me or UF in feed.

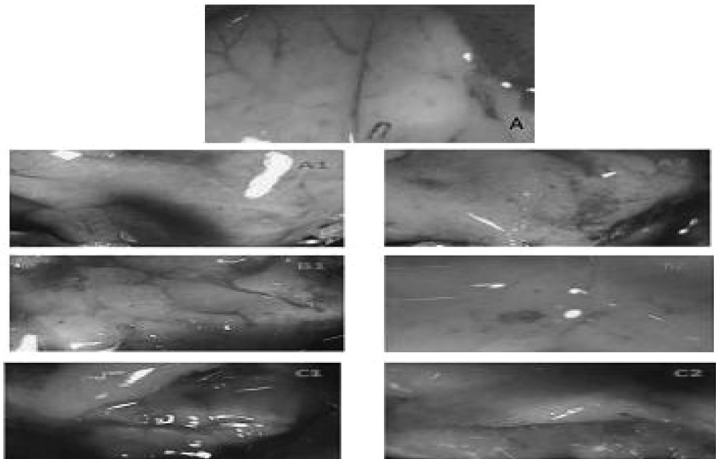

Figure 1 Gross lesions in the liver of tilapia fed with Me and UF combination diets. A: Control; A1, A2: Moderate diffuse hemorrhage of groups fed with 0.5% Me in combination with 0.5-2.0% UF in diets; B1, B2: Severe diffuse hemorrhage, brittle liver and necrosis of groups fed with 1.0% Me in combination with 0.5-2.0% UF in diets; C1, C2: Severe diffuse hemorrhage, congestion, liver enlargement, diffuse bile and necrosis of groups fed with 2.0% Me in combination with 1.0-2.0% UF in diets.

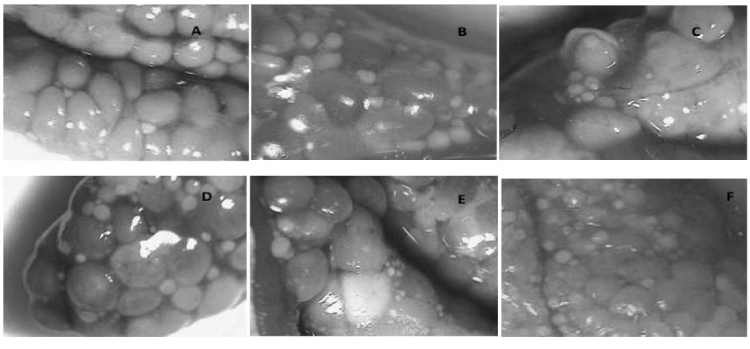

Figure 2 Gross lesions in the ovary of tilapia fed with Me and UF combination diets. A: Control; B-F: Groups fed with Me and UF diets which found ovary deformation, degeneration and various stages of ovary, abnormal shape of oocytes, nodules, erosion in the margin of some walls of oocytes, and liquefaction of the yolk.

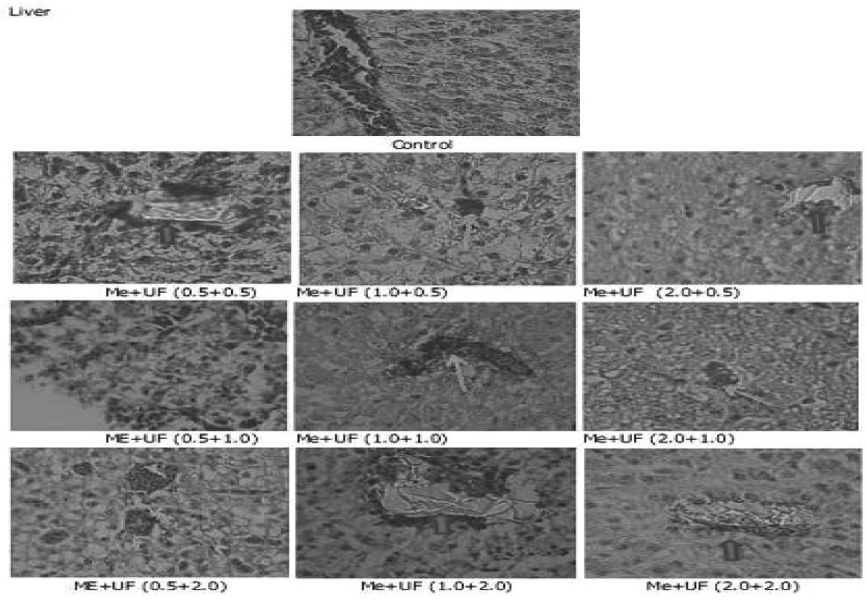

Figure 3 Histological view of crystals in the liver of tilapia fed with Me and UF combination diets. Liver degeneration, less prominent cell membrane, golden brown (long arrows) and transparent materials (short arrows) were detected under an inverted microscope (400X). The degree of degeneration, and the amounts and sizes of crystals were related to the levels of the combinations of Me and UF in diets.

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