Agr. Nat. Resour. 54 (2020) 9–16

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Comparison of frog diversity between paddy fields with chemical and non-chemical use in Nong Khai province, Thailand

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Article Info

Article history:
Received 14 June 2018
Revised 13 January 2019
Accepted 3 February 2019
Available online 28 February 2020

Keywords:
Anurans,
Chemical contaminants,
Frog compositions,
Frog species richness,
Rice field

Abstract

Rice is the most commonly cultivated crop in Southeast Asia and it has many natural pests. Fertilizers and pesticides are often applied to the paddy fields to encourage growth and to control pests; several publications have reported that these chemicals can have adverse effects on the physiology and development of anuran amphibians. However, until now, the effect of these chemicals on frog diversity in paddy fields has not been studied. Therefore, this study compared frog diversities between paddy fields involving chemical and non-chemical use. Four pairs of paddy fields (where each pair consisted of a paddy field using chemicals and a paddy field not using chemicals) in Nong Khai province, Thailand were selected as the study sites. The surveys were conducted once a month along five strip transects per paddy field site during June to November 2014. Although, chemical compounds were used in four chemical paddy fields, frog diversities were close and frog compositions were also similar between paddy field types. Moreover, the results of non-metric multidimensional scaling showed that frog compositions tended to be grouped according to the locations of study sites rather than the paddy field types. Although this study found similar frog assemblages between types of paddy fields, this also indicated that frogs dwelling in paddy fields cannot avoid chemical contaminants in the paddy field habitat. Therefore, long-term monitoring needs to be conducted to determine the accumulative effect of chemical contaminants on paddy field frog assemblages so that conservation planning can be appropriately developed for frogs dwelling in paddy fields.

Introduction

Hypotheses of the cause of global amphibian decline can be sorted into two major groups according to understanding the mechanism of each cause. Chemical contaminants are a cause that needs further studies to improve the understanding of the effect (Collins and Storfer, 2003). At a global scale, there are around 44 million rice farms, with rice being the most commonly cultivated crop in Southeast Asia (International Rice Research Institute, 2013a). Rice cultivation is exposed to many natural pests; therefore, to encourage growth and to control pests, fertilizers and pesticides are often applied in paddy fields. Moreover, water is usually trapped in the paddy field for a period of time during cultivation of the rice crop. The trapped water in the paddy field can attract amphibians from adjacent areas who need to keep their skin moist as well as requiring water to develop their young (Duellman and Trueb, 1994). Therefore, paddy fields are one amphibian habitat where the impact of chemical contamination on amphibians should be a concern.

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https://doi.org/10.34044/j.anres.2020.54.1.02

Chemical compounds such as pesticides used in agricultural areas can be at both lethal and sub-lethal toxicities depending on the amphibian. The sub-lethal effects on amphibians include developmental alteration, a sexual differentiation effect and increasing the susceptibility to disease (Mann et al., 2009). However, few researchers have focused on the chemical contamination effect on amphibian diversity which is linked to the functions of an ecosystem. Moreover, of the few studies focusing on chemical effects on amphibian assemblages were conducted in *ex situ* experiments (Boone and James, 2003; Relyea, 2005; Boone et al., 2007). Boone and James (2003) studied the interaction effects of an insecticide, herbicide and natural stressor on some amphibians. The populations of *Ambystoma maculatum* and *A. texanum* were virtually eliminated following insecticide treatment (carbaryl). Carbaryl also had a negative effect on the survival rate of *B. americanus*. The herbicide atrazine also had a negative effect on the body size, development and metamorphosis time of *Rana sphenocephala* and *B. americanus*. Moreover, they also reported that the small size and low developmental stage of the amphibians were influenced by the interaction of carbaryl and atrazine. Relyea (2005) reported that from a microcosm community which had five tadpole species, two species of tadpoles were completely eliminated, while a third species was nearly eliminated by glyphosate. The study on the interaction effect among chemical contamination, competition and predation on an aquatic amphibian community by Boone et al. (2007) showed that the survival rates of *B. americanus* and *A. maculatum* were reduced by the presence of multiple factors. The presence of these factors also prolonged the larval periods of *R. sphenocephala*. Although chemical compounds such as pesticides and fertilizers have been reported to be generally used in paddy fields and also have been reported to have an effect on amphibians, research is still lacking that focuses on the effect of the chemical compound on amphibian diversity in the paddy field, particularly based on *in situ* data. Therefore, a study involving the comparison of amphibian assemblages between paddy fields with chemical and non-chemical use should provide important information to improve understanding on the chemical contamination effect on amphibian assemblages in a paddy field habitat.

Thailand is one of the world's largest rice producers and exporters (International Rice Research Institute, 2013b). Nong Khai province is located close to the Mekong River and also has a high proportion of paddy fields compared to other agricultural types. Therefore, Nong Khai province was selected for the current study and the aim was to compare amphibian assemblages; diversities and compositions, between paddy fields with chemical and non-chemical use in Nong Khai province, Thailand. However, the amphibians reported in the study were only the two orders of Anura and Gymnophiona. In addition, based on the fact that all amphibians in the order Gymnophiona are fossorial species and only one species of Gymnophiona was reported in the study area, the primary focus in the study was on Anuran species.

Materials and Methods

Study site

Four study sites in Nong Khai province, Northeastern Thailand were sampled for frogs (Fig. 1). Each site was composed of a pair of paddy fields (one with chemical use and the other with no chemical use). Because of the rarity of non-chemical use in paddy fields, the first priority of the study site selection was to select four non-chemical fields and to then select adjacent fields where chemicals were used located. In each pair, the paddy fields were located between 1 to 2 km apart to avoid the effect of frog dispersion between the two paddy fields and also to achieve similar frog hunting intensity, weather and geographical conditions between the two paddy fields. The non-chemical paddy fields were in the past subjected to chemical use, but had been free of chemicals for several years (Table 1). The kinds and amounts of chemical compounds used in the chemical paddy fields are shown in Table 2.

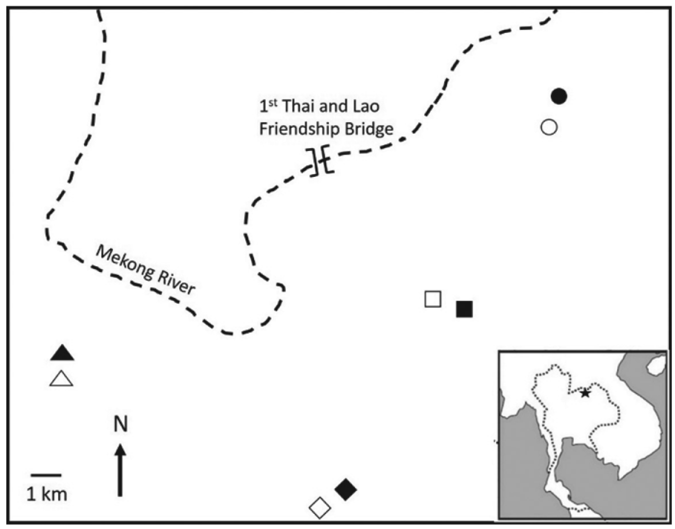

Fig. 1 Diagram of eight paddy fields where closed and open symbols indicate chemical and non-chemical use paddy fields, respectively, data represented for TB by (▲, △), for BP by (◆, ◇), for HK by (●, ○) and for HN by (■, □) and coordinates for TB, BP, HK and HN are Lat: 17.788° Long: 102.600°, Lat: 17.727° Long: 102.721°, Lat: 17.904° Long: 102.828°, and Lat: 17.814° Long: 102.775°, respectively

Table 1 Period without chemical compound use in non-chemical use paddy fields

Site name†	Period without chemical compound use (years)
Non-chemical use rice field at TB site	1
Non-chemical use rice field at BP site	3
Non-chemical use rice field at HK site	5
Non-chemical use rice field at HN site	6

† TB = study site at 17.788°, 102.600°; BP = study site at 17.727°, 102.721°; HK = study site at 17.904°, 102.828°; HN = study site at 17.814°, 102.775°.

Table 2 List of chemical compounds applied in chemical use paddy fields

Site name†	Name of chemical compound	Applied amount
Chemical use rice field at TB site	Fertilizer: 15-15-6 (NPK)	71.02 kg/ha
	Fertilizer: 15-15-0 (NPK)	142.04 kg/ha
	Herbicide (Pretilachlor): Chloroacetamide (30% weight per volume EC of 2-chloro-2',6'-diethyl-N-(2-propoxyethyl) acetanilide)	4.26 L/ha
Chemical use rice field at BP site	Fertilizer: 15-15-15 (NPK)	156.25 kg/ha
Chemical use rice field at HK site	Fertilizer: 15-0-0 (NPK)	156.25 kg/ha
	Fertilizer: 20-20-20 (NPK)	156.25 kg/ha
	Surfactant: Nonylphenol ethoxylates (60% by weight of poly(oxy-1,2-ethanediyl), .alpha.-(nonylphenyl)-.omega.- hydroxy-)	0.78 L/ha
Chemical use rice field at HN site	Fertilizer: 15-15-15 (NPK)	156.25 kg/ha
	Herbicide (2,4-d butyl ester + Butachlor): phenoxycarboxylic acid + chloroacetamide (3.76% of butyl (2,4-dichlorophenoxy) acetate + N-butoxymethyl-2-chloro-2',6'-diethylacetanilide + 4% granular)	7.21 kg/ha

† TB = study site at 17.788°, 102.600°; BP = study site at 17.727°, 102.721°; HK = study site at 17.904°, 102.828°; HN = study site at 17.814°, 102.775°.

Frog sampling

Since frogs in the area surrounding the paddy field could be attracted by the trapped water in the paddy field, the frogs attracted should be ephemeral pond breeders which have high activity in the rainy season. The farmers were also very active in the paddy field during the middle of the rainy season until early in the dry season. Thus, all eight paddy fields in this study were surveyed at least once a month from June to November 2014 which covered both the rainy season and farmer activity in the study area. Five strip transects (10 m × 2 m) were sampled in each paddy field. All strip transects were surveyed for frogs at night (1930–2300 hours) once a month using the active survey method (Heyer et al., 1994). At the same paired study site, frog surveys were conducted in the chemical and non-chemical use paddy fields during the same night to achieve similarity in weather conditions. All frogs found in the strip transects were identified to the species level and the number of individuals was also recorded for each sampling night.

Data analysis

Significant differences ($p < 0.05$) of the abundance of each species between chemical and non-chemical use paddy fields were determined using a Mann-Whitney U-test, in the R version 3.1.2 software package (R Core Team, 2013). Frog species diversity and composition were determined based on the data of frog species and number of each species in each paddy field. The difference in species diversities between chemical and non-chemical use paddy fields were determined using the rarefaction curve at the 95% confident interval (CI) according to Magurran (2004). Frog species compositions were compared between chemical and non-chemical use paddy fields using non-metric multidimensional scaling (NMDS) and significant differences in frog composition between chemical and non-chemical use paddy fields were determined using analysis of similarity (ANOSIM). The percentages of similarity in species compositions between chemical and non-chemical use paddy fields were determined using the Morisita-Horn index (Colwell, 2013). The correlations between frog assemblage and the used amount of chemical compounds in paddy fields were determined by fitting the variables onto the NMDS plot. NMDS plot, ANOSIM testing and fitting the amounts of chemical variables onto the NMDS plot were generated by the R version 3.1.2 software package with the vegan package (Oksanen et al., 2015). Rarefaction values with the 95% CI and the Morisita-Horn index were calculated using EstimateS version 9.1.0 (Colwell, 2013). The rarefaction values with the 95% CI were plotted using R version 3.1.2 software package.

Results

The farmer activities in all sampled paddy fields were similar in all aspects with the exception of chemical type based on aspect. Chemical compounds, including fertilizers, herbicides and surfactants, were used in the chemical paddy fields whereas they were not in the non-chemical use paddy fields (Table 2). Among the three types of chemical compounds, fertilizers were the most frequently used and were used in all chemical fields. Herbicides were used in two paddy fields (HN and TB sites) and surfactant was used only in one paddy field (HK site).

During the study period, 127 frogs and 7 frog species were found—*Occidozyga lima*, *O. martensii*, *Fejervarya limnocharis*, *Hoplobatrachus rugulosus*, *Hylarana macrodactyla*, *Microhyla butleri*, and *M. fissipes*. The abundance (number of frog occurrences per month) of each frog species are shown in Figs. 2A–H. Although the frog abundance of each species varied among the study sites and between the paddy field types, the results from the Mann-Whitney U-test indicated that there were no significant differences in frog abundance between chemical and non-chemical use paddy fields for all study sites (Table 3). *H. rugulosus* was the only species found in non-chemical use paddy fields (TB and BP sites) but it was not found in chemical use paddy fields (Fig. 2D). However, the abundance of *H. rugulosus* in non-chemical use paddy fields was low; therefore, there was no significant difference in abundance of *H. rugulosus* between non-chemical and chemical use paddy fields.

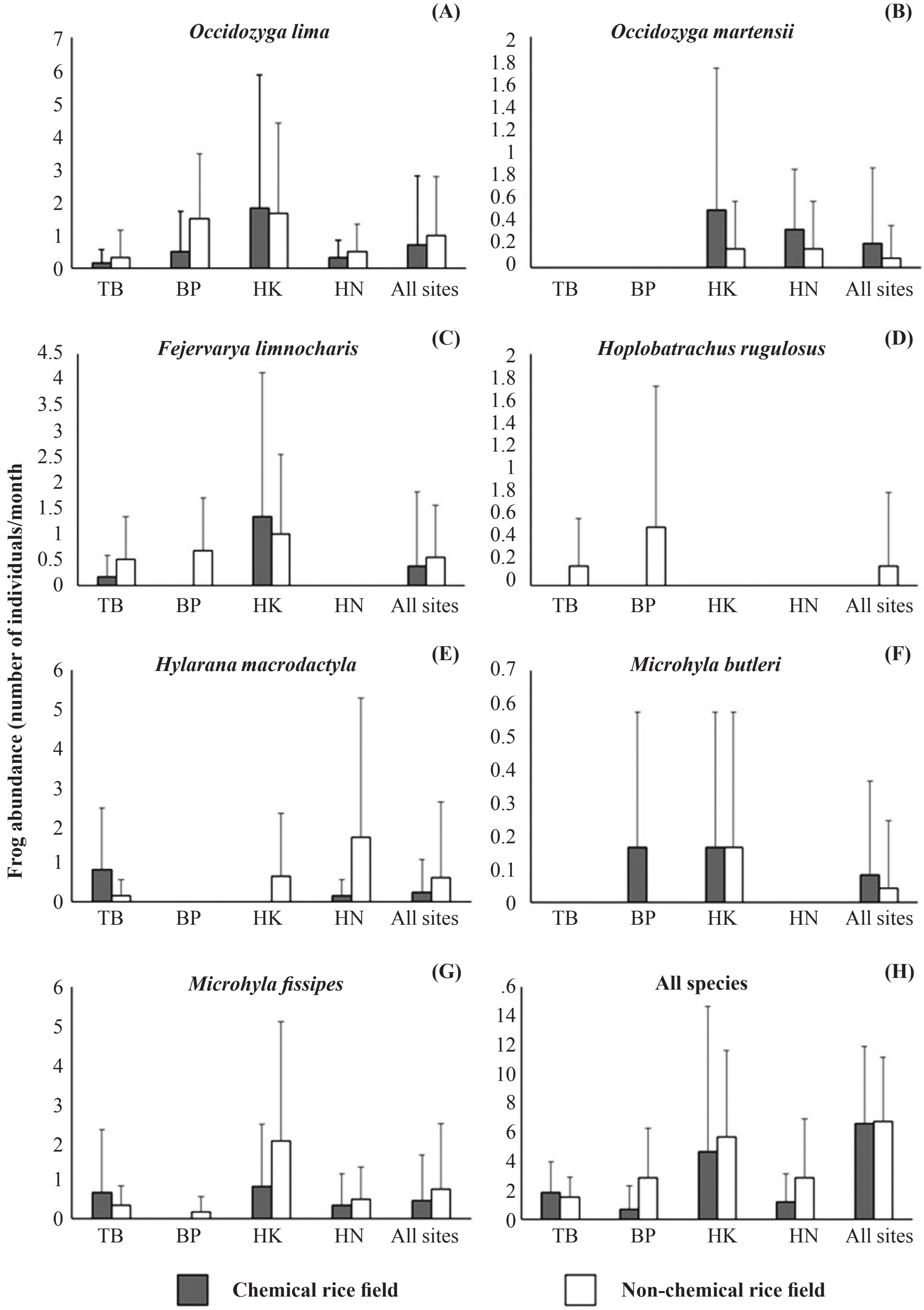

Fig. 2 Abundance of each frog species in chemical and non-chemical use paddy fields: (A) *Occidozyga lima*; (B) *Occidozyga martensii*; (C) *Fejervarya limnocharis*; (D) *Hoplobatrachus rugulosus*; (E) *Hylarana macrodactyla*; (F) *Microhyla butleri*; (G) *Microhyla fissipes*; (H) all species, where error bars = Standard error

Table 3 Mean and SD of abundance of each frog species in chemical and non-chemical use paddy fields

Species	Initial species name	Paddy field types	Site† TB	BP	HK	HN	All sites
			Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Occidozyga lima	OcLi	Chemical	0.17 ± 0.41	0.50 ± 1.22	1.83 ± 4.02	0.33 ± 0.52	0.71 ± 2.10
		Non-chemical	0.33 ± 0.82	1.50 ± 1.97	1.67 ± 2.73	0.50 ± 0.84	1.00 ± 1.77
Occidozyga martensii	OcMa	Chemical	-	-	0.50 ± 1.22	0.33 ± 0.52	0.21 ± 0.66
		Non-chemical	-	-	0.17 ± 0.41	0.17 ± 0.41	0.08 ± 0.28
Fejervarya limnocharis	FeLi	Chemical	0.17 ± 0.41	-	1.33 ± 2.80	-	0.38 ± 1.44
		Non-chemical	0.50 ± 0.84	0.67 ± 1.03	1.00 ± 1.55	-	0.54 ± 1.02
Hoplobatrachus rugulosus	HoRu	Chemical	-	-	-	-	-
		Non-chemical	0.17 ± 0.41	0.50 ± 1.22	-	-	0.17 ± 0.64
Hylarana macrodactyla	HyMa	Chemical	0.83 ± 1.60	-	-	0.17 ± 0.41	0.25 ± 0.85
		Non-chemical	0.17 ± 0.41	-	0.67 ± 1.63	1.67 ± 3.61	0.63 ± 1.97
Microhyla butleri	MiBu	Chemical	-	0.17 ± 0.41	0.17 ± 0.41	-	0.08 ± 0.28
		Non-chemical	-	-	0.17 ± 0.41	-	0.04 ± 0.20
Microhyla fissipes	MiFi	Chemical	0.67 ± 1.63	-	0.83 ± 1.60	0.33 ± 0.82	0.46 ± 1.18
		Non-chemical	0.33 ± 0.52	0.17 ± 0.41	2.00 ± 3.10	0.50 ± 0.84	0.75 ± 1.70
All species		Chemical	1.83 ± 2.14	0.67 ± 1.63	4.67 ± 9.99	1.17 ± 1.94	6.58 ± 5.33
		Non-chemical	1.50 ± 1.38	2.83 ± 3.43	5.67 ± 5.96	2.83 ± 4.07	6.71 ± 4.45

† TB = study site at 17.788°, 102.600°; BP = study site at 17.727°, 102.721°; HK = study site at 17.904°, 102.828°; HN = study site at 17.814°, 102.775°.

Regarding frog species richness, six and seven species were found in chemical and non-chemical use paddy fields, respectively. However, the results from the rarefaction curves with the 95% CI indicated that there was a large overlap between the CI curves for chemical and non-chemical use (Fig. 3A), indicating that there was no significant difference in species richness between the chemical and non-chemical use paddy fields. Frog species richness between paddy field types was also compared at each study site. The rarefaction curves with the 95% CI of chemical and non-chemical use paddy fields also a large overlap between the 95% CI curves at all sites (Figs. 3B–E). These results showed that for either the combined data from all the study sites or the data from each study site, the frog species richness was not significantly different between chemical and non-chemical use paddy fields.

Frog species composition was determined using the NMDS plot with stress = 0.05; it indicated that chemical and non-chemical use paddy fields were not suited to be grouped according to paddy field type (chemical and non-chemical use), whereas there was a trend supporting grouping by location of the study sites (Figs. 4A and 4B). This indicated that the frog compositions between chemical and non-chemical use paddy fields were similar. The analysis of similarity (ANOSIM) also indicated that frog assemblages were not significantly different between chemical and non-chemical use paddy fields (p = 0.706). Furthermore, the Morishita-Horn similarity index indicated that frog compositions between chemical and non-chemical use paddy fields had very high similarity (96.5%).

The correlation between the NMDS scores and variables related to the amounts of chemical used was not significant (Fig. 5). This indicated that the occurrence of frogs in the eight paddy fields was not significantly correlated with the amounts of chemical used in the paddy fields.

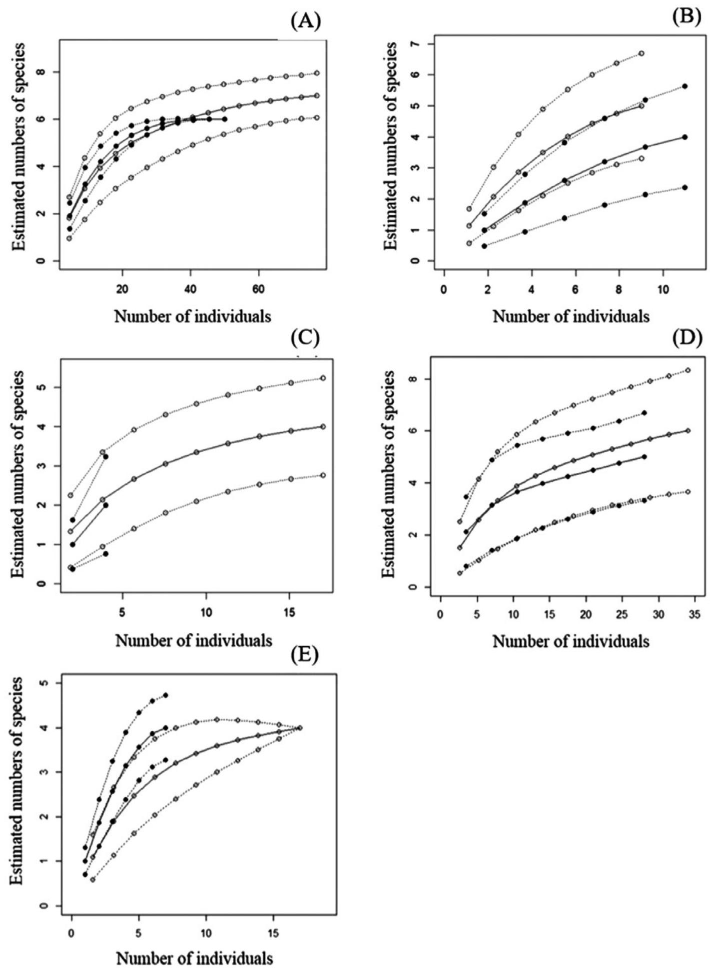

Fig. 3 Rarefaction curves with the 95% confidence interval (CI) of frog species richness between chemical and non-chemical use paddy fields, where closed and open dots indicate chemical and non-chemical use paddy field data, respectively, solid and dashed lines indicate rarefaction values and the 95% CI, respectively: (A) overall site data; (B) TB site data; (C) BP site data; (D) HK site data; (E) HN site data

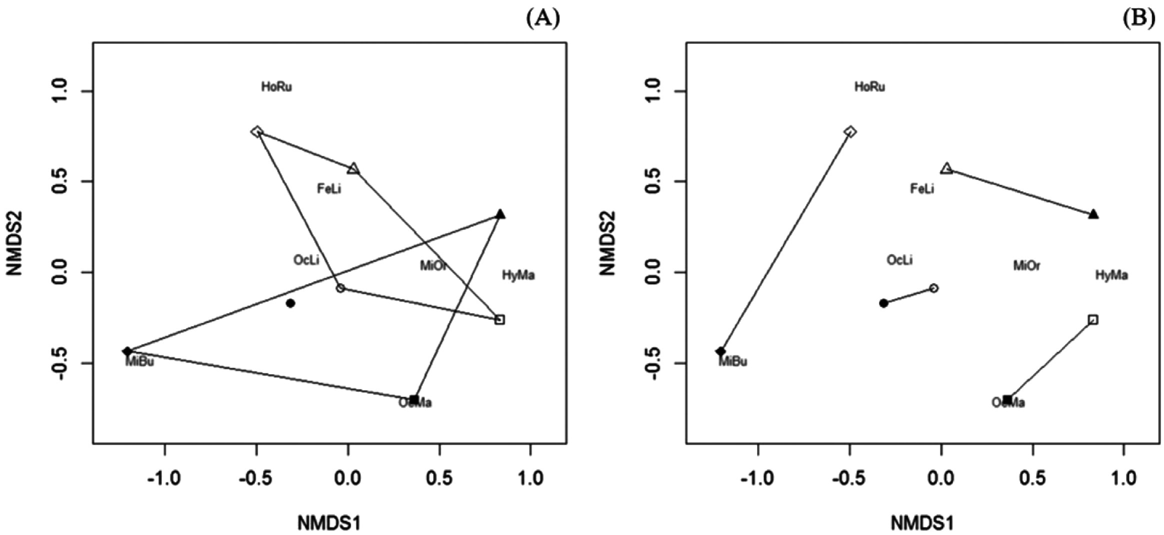

Fig. 4 NMDS plot of frog assemblages generated based on data from eight paddy fields (Stress = 0.049), where closed and open dots indicate chemical and non-chemical use paddy field data, respectively, data represented for TB by (▲, △), for BP by (◆, ◇), for HK by (●, ○) and for HN by (■, □) and initial species names are provided in Table 3: (A) Solid polygon grouping the data according to chemical and non-chemical use paddy fields; (B) solid line grouping the data according to study site locations

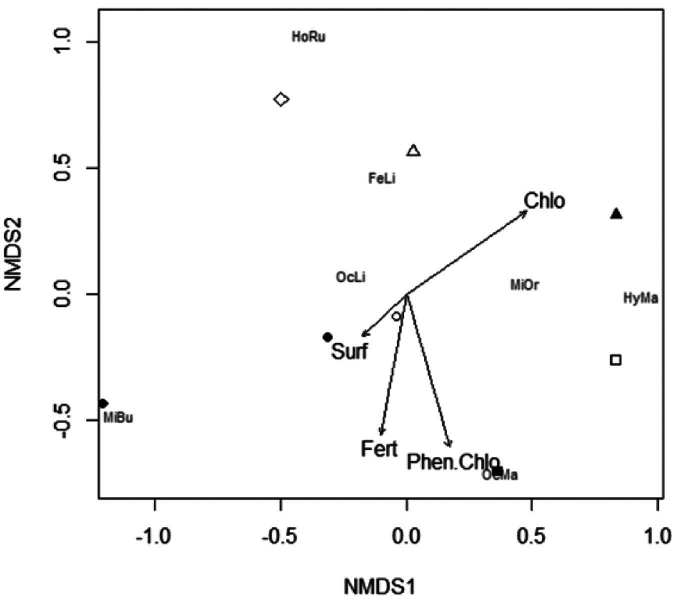

Fig. 5 NMDS plot of frog assemblages generated based on data from eight paddy fields (Stress = 0.049), where closed and open dots indicate chemical and non-chemical use paddy field data, respectively, data represented for TB by (▲, △), for BP by (◆, ◇), for HK by (●, ○) and for HN by (■, □), initial species names are provided in Table 3and chemical compounds applied in paddy fields are shown as Fert, Surf, Phen.ChloFertilizer and Chlo for Fertilizers, Surfactant, Phenoxycarboxylic acid+Chloroacetamide and Chloroacetamide, respectively

Discussion

Chemical compound and frog diversity

Three types of chemical compounds (fertilizers, herbicides and surfactants) were found at the study sites. Fertilizers were found in all chemical paddy fields. There were several fertilizer formulas applied on the study sites, with inorganic nitrogenous compounds found in all formulas (Table 2). Pretilachlor and 2,4-d butyl ester + Butachlor were the herbicides used and were found in two paddy fields. Nonylphenol ethoxylate was the surfactant used and was found only in one paddy field.

As fertilizers had been applied in all chemical use paddy fields in the study (Table 2), inorganic nitrogenous compounds (ammonia: NH_3/NH_4^+; nitrite: NO_2^-; nitrate: NO_3^-) would be expected to be present in the water in all chemical use paddy fields. Ammonia has the highest toxicity follow by nitrite and nitrate is the least toxic; the lethal effect of nitrogenous compounds in several amphibian species has been commonly reported (Mann et al., 2009). *Fejervarya limnocharis* was also reported to be affected by nitrate and for this species the median LC50 dose (the dose required to kill half the members of a tested population after a specified test duration) was a concentration of 33.7 mg/L within 96 hr.

In the current study, the herbicide Butachlor (0.4–0.8 mg/L) had a negative effect on the survival, development and metamorphosis time of tadpoles, and on the DNA of *F. limnocharis*. In addition, 2,4-dichlorophenoxyacetic acid (2,4-D) + Butachlor at low concentrations (2,4-D at 25–75 parts per million (ppm) and Butachlor at 1–2.5 ppm) significantly increased the frequency of micronuclei and altered cells of erythrocytes of catfish (*Clarias batrachus*) according to Ateeq et al. (2002).

Nonylphenol ethoxylates (NPEs) are widely used as surfactants in agriculture and also industrially (Bernabò et al., 2014). Bernabò et al. (2014) reported that NPEs have a negative effect on the liver of newts even at low concentrations (50 and 100 μg/L) and for a short time of exposure (96 hr).

Although fertilizers, herbicides, and surfactants were applied as chemical in paddy fields and negative effects of these chemicals on amphibians have been reported, the current results indicated that frog diversities were not significantly different between chemical and non-chemical use paddy fields and the frog composition between the two paddy field types were very similar (96.5%) and the paddy field points from NMDS plot were not grouped according to the type of paddy field. Moreover, the ANOSIM analysis also indicated that there were no significant differences in frog compositions between the two paddy field types. This indicated that in the study areas, frog assemblages cannot be used to show the difference between non-chemical and chemical use paddy fields.

However, from the NMDS plot of the BP site, the chemical use field point was located far from the non-chemical use field point (Fig. 4B), indicating that the species composition between the two paddy field types at BP site were quite different. Although the result indicated a difference in frog composition at the BP site, this may not have been the result of chemical contamination because the chemical use paddy field at the BP site had the lowest number of chemical compound types and there was only one type of chemical compound used in that paddy field, namely a fertilizer with formula 15-15-15 for N-P-K (Table 2). In addition, fertilizer with exactly the same formula as that applied at the BP site was applied in the chemical use paddy field at the HN site and a similar formula was also applied in other chemical paddy fields at all sites, while the frog compositions at all the other sites were similar between non-chemical and chemical use paddy fields. This indicated that the difference in frog composition between paddy field types at the BP site should not be associated with the effect of chemical contamination.

Therefore, it can be simply concluded based on the current study that although fertilizers, herbicides, and surfactants were applied in the chemical use paddy fields and the negative effects of these chemicals on amphibians have been reported previously, the frog assemblage in the current study did not show any significant differences between chemical and non-chemical use paddy fields. Although the results seem to indicate that frog assemblages were not affected by the chemical compounds applied in the paddy fields, there are some aspects identified from this study which should be of concern.

First, paddy field dwelling frogs may not be highly sensitive species. All study areas sampled in the current study for both chemical and non-chemical use were not natural habitats. Other frog species which are highly specific to the natural habitat should not exist in both non-chemical and chemical paddy fields. Therefore, the results from this study cannot be used to conclude that there is a chemical effect on all natural frog assemblage; the results are relevant only to the chemical effect on paddy field frog assemblage for sites that do not have a natural frog community.

Another aspect of concern was related to the size of the non-chemical use paddy fields. Due to the non-chemical use paddy fields in this study being surrounded by chemical use paddy fields, the size of the non-chemical use paddy field may not have had a large enough core area to buffer it from the effects of chemicals used in adjacent paddy fields. Some frogs which are highly sensitive to chemical compound may not have survived even in the non-chemical use paddy field. Moreover, frogs are mobile and it is very likely that individuals may frequently move in and out of particular paddy fields from adjacent ditches and streams. This immigration and emigration would likely tend to even out the frog diversity among all the paddy fields in a large area. Without enclosing each paddy field selected for measurement, immigration and emigration remain uncontrolled. These reasons may have been responsible for this study not detecting any significant differences in frog assemblages between non-chemical and chemical use paddy fields.

Information for conservation planning

The current results did not show any significant differences in frog community and population levels between non-chemical and chemical use paddy fields. This may indicate that frogs in this study did not have the ability to avoid the chemical compounds applied in the paddy fields. This would mean that all frog species may have been receiving the chemical compounds even at low doses but in the long term, this might have affected future frog assemblage and abundance. Although all frog species identified in this study were listed as on the least concern level in the International Union for Conservation of Nature (IUCN) red data list, the major threat, noted by specialists from the IUCN, to most species (five of the seven species—*F. limnocharis*, *H. rugulosus*, *H. macrodactyla*, *M. butleri* and *M. fissipes*) was pollution in agricultural area (Diesmos et al., 2004; van Dijk et al., 2004a; van Dijk et al., 2004b; van Dijk et al., 2004c; Lau et al., 2008; van Dijk et al., 2009a; van Dijk et al., 2009b). Moreover, one species (*M. butleri*) has been reported to have not been found in paddy fields in Taiwan because of the history of pesticide and fertilizer application there. Therefore, another aspect that should be of concern for conservation planning regarding these five species would be the long term effect of chemical contamination to the paddy field habitat.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors sincerely thank Ms Jirarat Jiyawat, Mr Bunpeng Nampim, Mr Teerapong Suticha, Mr Pow Butharakot, Ms Chalao Bunhao, Ms Anong Worana, Mr Chotwit Wohandageworanan, Ms Charoenrat Wohandageworanan and Ms Kun Photiphaitoon for providing the study sites. The project funding was supported by Khon Kaen University.

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