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Implication Pesticide Residues on Abundance and Diversity of Anurans in Transplanted Rice Crop

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Abstract: During present study, six anuran species which belonged to order anura and families bufonidae and dicroglossidae from transplanted rice crop fields during 2021 and 2022. The species included *Duttaphrynus melanostictus, D. stomaticus, Euphlyctis cyanophlyctis, E. adolfi, Hoplobatrachus tigerinus* and *Fejervarya limnocharis* among which *E. adolfi* had patchy occurrence, whereas *E. cyanophlyctis* was most abundant species Analysis of monthly differences of anuran abundance at all study sites showed July and August as high peak activity periods in all rice fields, about 13% increase in anuran abundance was during 2022 as compared to previous year. However, 36% increase in anuran population was recorded in unsprayed as compared to sprayed transplanted rice crop fields. Overall increase in body weights of male and female anuran species was 14.21 and 11.17%, respectively in unsprayed as compared to sprayed fields. Positive correlation was observed between amphibian population with atmospheric temperature, relative humidity and rainfall. Pesticide residues were below the limit of quantification (0.01 mg kg^{-1}) in soil, excretory contents and water samples without any amphibian morphological abnormality. The study suggests rice agroecosystems, in partnership with neighboring natural aquatic and terrestrial habitats without use of pesticides can serve as a sanctuary for anuran biodiversity.

Keywords: Abundance, Anuran, Diversity, Frog, Pesticide residue, Rice crop

Expansion and intensification of agricultural land use are the most significant anthropogenic changes in the last century (Pascual and Perrings 2007). All long-term land use changes resulted in the conversion of natural ecosystems to seminatural or artificial systems which have negatively affected biodiversity composition and ecological processes (Kanianska 2016). Land use changes often likely resulted in decreased species diversity as changes in forest cover, vegetation type and composition, soil characteristics and topography will provide different microhabitats and resources for wildlife than existed prior to destruction. In Punjab, paddy accounts for 87% of total kharif crop area which during 2021-22 occupied 31.45 lakh hectares with total paddy production of 203.71 lakh tonnes. In addition to providing a necessary food source, irrigated rice cropping systems also serves as artificial wetland for a variety of species including amphibians (Natuhara 2013). Rice agro-ecosystem creates a landscape mosaic of hydroperiods, which improves the richness of regional species. The usage of rice fields as a substitute for natural wetlands may be contingent on management practises such as the use of agrochemicals or organic agriculture, irrigation water source and offseason crop area use (Donald 2004). Amphibian species forage and breed in rice fields and adjacent irrigation ditches (Frost et al., 2008). In terms of diversity, amphibians play a key role in many natural ecosystems and greatly contributes to the global biomass. Among vertebrates, amphibians (41%) is the most threatened group with extinction as compared to mammals (27%) and birds (13%) (IUCN 2021) which breathe through lungs (in adult stage) and gills (in tadpole stage) thus, hold a key position in the ecosystem as 'ecological indicators' (Simon et al., 2011). In rice agro-ecosystem, amphibians play important role in pest control and in enhancing soil fertility and productivity. Many factors are responsible for their decline, such as climate change, habitat destruction, pollution, deforestation, infectious diseases, heavy metals, pesticide contamination and UV radiation exposure (Blaustein et al., 2003). At global level, habitat modification is a key factor which contributes to amphibian declines with an estimated 63% of all amphibian species affected and approximately 87% of the threatened amphibian species affected (Chanson et al., 2008). Overuse of pesticides, field water contamination with heavy metals agricultural fields especially rice fields are not suitable habitat for amphibian species (Bruhl et al., 2013),as these possess the potential to interfere with physiology, growth, behaviour and regulation of reproductive, developmental processes and alterations in the neurological and immune system of the organism (Andreia et al., 2019). The effect has been seen on clutch size, larval survival rate hence, directly affecting the population trajectories of amphibians. Loss of habitat and increased pesticide inputs are two significant factors that have a synergistic effect in the contribution of reduction in amphibian population (Stuart et al., 2004, Kiesecker 2010).

In India, due to the patchy and fragmented amphibian species distribution very little information is available at the population, abundance and diversity levels. They have neither been adequately recorded nor monitored over time in relation to extinction risks, population vulnerability and human-induced changes (Aravind et al., 2004). During the last decade alone, there has been an increase in the discovery of amphibians across India which has led to the reports of higher amphibian species diversity and description of as many as 100 amphibian species (Hebbar et al., 2019). The present study was conducted for two seasons to analyse the abundance, diversity and effect of any pesticide residue on amphibian species in rice crop fields.

MATERIAL AND METHODS

Study area: The present study was carried out in rice crop fields of six selected villages of district Ludhiana during 2021 and 2022. Ludhiana is the largest district located in the central region of Punjab (India), having tropical steppe, hot and semi-arid climate with very hot summers and chilly winters. The south-west monsoon, which arrives during final week of June and departs towards the end of September, accounts for around 78% of annual rainfall. Rice crop fields having two different management practices were selected: (1) with use of pesticides to control insect pests and diseases and use of chemical fertilizers to fulfil nutritional requirements as per recommended doses (2) unsprayed rice crop fields, where application of any pesticide was prohibited and nutritional requirements were fulfilled by the use of farm yard manure.

Sprayed rice crop fields include application of herbicides such as pretilachlor (500 grams per acre acre) before and after transplanting rice crop for the control of grasses and weeds and application of insecticides like cartap hydrochloride , emamectin benzoate and carbendazium for the control of insect-pests whereas fungicides like tebucoazole and veldamycin were sprayed to control diseases. In unsprayed rice crop fields, no pesticide and chemical fertilizers were applied on rice crop from the last 10 years. The climatic factors like atmospheric temperature (^{0}C), rainfall (mm) and relative humidity (%) and the edaphic factors like soil pH, electrical conductivity and organic carbon content were also recorded and correlated with the amphibian population.

Data collection: Visual encounter survey method (VESM) was applied for estimating the anuran population. For estimation of individuals from rice crop fields, each plot consists of 0.4 ha area with three replications. The count of individuals from each plot was recorded from four corners and centre as five belt transects with size of each belt transect as 50×4 m. All observations were recorded at fortnight intervals (pooled at month level) from May to October months during 2021 and 2022, mostly during early morning 06:00 am to 08:00 am. Capturing of anurans from water was done using scoop net and each specimen was checked for any morphological abnormalities and was released back in their natural habitat. Anurans were identified by using respective identification keys Daniel and Seakar (1989), Daniel (2005) and from ZSI (Zoological Survey of India). Different indices like Simpson's index, Shannon-Weiner index, species evenness and species richness were calculated. Each anuran was measured for its body weight and morphometric measurements (mm) using a digital vernier calliper (Themisto® TH-M61). Spearman's correlation was employed to analyse the anuran community (richness and abundance) versus ambient temperature, rainfall and relative humidity.

Sampling of soil and excretory contents: From the selected sprayed transplanted rice crop fields, the soil samples were collected after spray of different pesticides on rice crop at 7-day interval. Uniform slices of soil samples were taken out separately and packed in cloth bags and related information like date, location, time, crop, depth, sample number was labelled with the help of marker. Urine and faecal matter samples were taken using Krogh (1973) and Bennett (1999) methods, respectively from anurans of sprayed and unsprayed transplanted rice crop fields at monthly intervals. Urine and faecal samples were collected in plastic vials and plastic packets, respectively and deep freeze at -4°C.

Extraction and clean-up procedure: The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method for estimating pesticide residues was used to prepare the soil and faecal samples. Liquid-liquid phase extraction (LLE) method was used to prepare water and urine samples.

Estimation by GLC: Different soil and excretory content samples were estimated for any pesticide residues using Gas Liquid Chromatography (Shimadzu model GC-2010) equipped with ECD/FTD supplied by M/S Shimadzu (Japan) in Toxicology laboratory, Department of Entomology, Punjab Agricultural University, Ludhiana.

Estimation by HPLC: Soil samples were estimated for pesticide residues using High Performance Liquid Chromatograph (HPLC): Shimadzu (Model DGU-2045) equipped with reversed-phase C18 column and PDA detector supplied by M/SShimadzu (Japan) in the Toxicology laboratory, Department of Entomology, Punjab Agricultural University, Ludhiana. The HPLC analysis was carried out at column temperature 25°C under isocratic condition acetonitrile/water (80/20, v/v) with pump flow at 0.4 mL min^{-1}.

Recovery studies: Recovery experiments were implemented to evaluate the competence of the analytical method used. Samples of soil and excretory contents were spiked with pesticides at 0.05, 0.0005 and 0.01 mg kg^{-1} levels, respectively. The reagent blank was also processed in the same way so as to find out the interference, if any due to reagents. The following formula was used for quantification of pesticide residues:

$$\text{Residue(ppm)} = \frac{\text{ng of standard injected}}{\text{Area of standard}} \times \frac{\text{Area of sample}}{\mu\text{L of sample injected}} \times \frac{\text{Final Volume of sample (mL)}}{\text{Weight of sample (g)}}$$

RESULTS AND DISCUSSION

Diversity and abundance of anurans in rice crop fields: **T**otal of 3945 individuals of six anuran species were recorded from the transplanted rice crop fields (sprayed and unsprayed) which belonged to order anura; and families bufonidae and dicroglossidae during 2021 and 2022 (Table 1). Recorded species included *Duttaphrynus melanostictus* (Schneider 1799)*, D. stomaticus* (Lütken 1864)*, Euphlyctis cyanophlyctis* (Schneider 1799)*, E. adolfi* (Gunther 1860)*, Hoplobatrachus tigerinus* (Daudin 1802) and *Fejervarya limnocharis* (Gravenhorst 1829) among which *E. adolfi* had patchy occurrence and were present at Sites A, D and F only. *E. cyanophlyctis* was the most abundant species in all the selected sites as it remains active during whole year, whereas other species were active during summer (May, June) or monsoon (July, August) season. The incidence of all the six anurans took place during May to October. Analysis of monthly differences of anuran abundance at all study sites showed July and August as high peak activity periods (Fig. 1) as these months received maximum precipitation due to which there is enough water in crop fields and other water bodies which along with suitable temperature leads to congenial environmental conditions for the survival and reproduction of anurans. Site D was most diverse whereas Site B least diverse as indicated by Shannon-Wiener index (H') and Simpson diversity index (D) (Table 2). Higher species evenness recorded at Site C indicated that anuran species were more evenly distributed as compared to other sites.

In sprayed rice crop fields, mean anuran populations in 2022 increased by 3.85% at Site A, 13.92% at Site B, and 5.34% at Site C compared to 2021. *E. cyanophlyctis* was the most abundant species across all sites. The least abundant species varied by site: *E. adolfi* at A, *D. melanostictus* at B, and *F. limnocharis* at C, all showing statistically significant

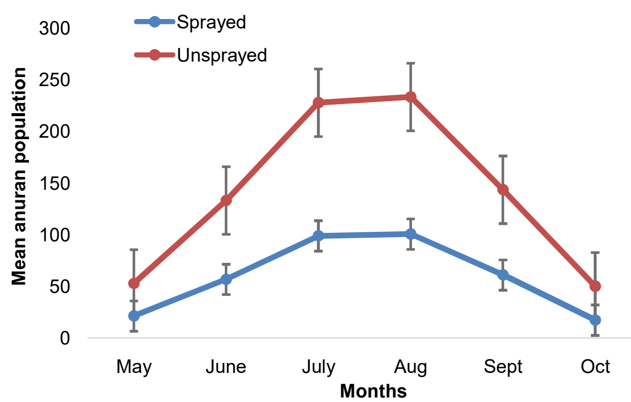

Fig. 1. Relative incidence of anurans in sprayed and unsprayed transplanted (2021 and 2022) rice crop fields

Table 1. Inventory of anuran species recorded in transplanted rice crop fields

Common name	Scientific name	Family	Order	IUCN status
Common Asian toad	*Duttaphrynus melanostictus* (Schneider 1799)	Bufonidae	Anura	Least concern
Indian marble toad	*Duttaphrynus stomaticus* (Lütken 1864)	Bufonidae	Anura	Least concern
Indian skittering frog	*Euphlyctis cyanophlyctis* (Schneider 1799)	Dicroglossidae	Anura	Least concern
Adolf's speckled frog	*Euphlyctis adolfi* (Gunther 1860)	Dicroglossidae	Anura	Least concern
Rice field frog	*Fejervarya limnocharis* (Gravenhorst 1829)	Dicroglossidae	Anura	Least concern
Indian bull frog	*Hoplobatrachus tigerinus* (Daudin 1802)	Dicroglossidae	Anura	Least concern

Table 2. Alpha diversity indices for anurans in different selected sites in rice crop fields

Biodiversity indices	Sprayed rice crop fields			Unsprayed rice crop fields		
	Site A	Site B	Site C	Site D	Site E	Site F
Shannon-Wiener index	1.738	1.598	1.606	1.771	1.601	1.762
Simpson diversity index	0.182	0.205	0.202	0.173	0.203	0.176
Index of similarity	0.818	0.795	0.798	0.827	0.797	0.824
Species evenness	0.970	0.993	0.998	0.988	0.995	0.983
Species richness	6.0	5.0	5.0	6.0	5.0	6.0

differences. In unsprayed rice crop fields, mean anuran populations in 2022 rose by 13.51% at Site D, 8.07% at Site E, and 10.44% at Site F compared to 2021. *E. cyanophlyctis* remained the most abundant species across all sites. The least abundant species were *E. adolfi* at Sites D and F, and *D. melanostictus* at Site E, all statistically distinct from others. Among sprayed rice fields, Site A (Noorpur Bet) was the most diverse, while Site B (Ladian Kalan) showed the highest population increase in 2022 due to nearby water bodies supporting breeding and food availability. In unsprayed fields, Site F (Burj Lambran) was most diverse, but Site D (PAU) had the highest population increase. Overall, anuran abundance was greater in unsprayed fields, with a 36.03% higher mean increase due to more breeding sites and insect food availability compared to pesticide-treated fields.

No significant difference in anuran species diversity was observed between sprayed and unsprayed rice fields, suggesting that landscape structure plays a more crucial role than farming practices. These rice crop fields are subjected to a variety of agricultural management practises, including the degree of vegetation conservation at field edges, crop rotation, agrochemicals and their mode of application, the management level, the availability of uncultivated regions in the field and the surrounding landscape environment (Attademo et al., 2005) which directly or indirectly affect anuran guilds. Overall, pesticide-free agriculture supports greater biodiversity by reducing environmental stressors, though landscape elements may exert a stronger influence than the type of crop management applied. Vegetation around the rice fields can have an impact on amphibian population sizes in natural environments (Weibull et al., 2000).

Environmental cues like temperature, moisture or the timing and amount of precipitation are the main components that affect anuran phenology directly. Inadequate rainfall, extreme drought and shortened hydro-periods have all been linked to a decrease in anuran calling activity, catastrophic reproductive failure in many pond-breeding amphibians, metamorphosis at smaller body sizes, the potential local elimination of paedomorphosis and local extinctions. Pounds et al. (2006) provided an illustration of the intricate linkages between anuran population losses and changes in the ecosystem at large scale.

Body weights and morphometric measurements: The observed body weights of anuran species from unsprayed transplanted rice crop fields were more as compared to sprayed transplanted rice crop fields which may be due to more food availability and reduced toxic stress. In unsprayed habitats, the abundance of insects and other invertebrates provides ample nutrition, which directly supports better growth, body condition, and reproductive fitness in frogs and toads. Conversely, pesticide application in sprayed fields reduces the diversity and density of prey organisms, thereby limiting food resources (Table 4). In case of *D. melanostictus, D. stomaticus, E. cyanophlyctis, F. limnocharis* and *H. tigerinus* the males exhibited increase in body mass ranging from 0.81-10.63% heavier, while females ranging from 0.21-15.90% during 2021 and 2022. The maximum difference was recorded in case of *E. adolfi* males and females during the study period which indicates this species may be particularly sensitive to pesticide exposure, showing a stronger negative impact on growth. The overall, increase (%) in SVL, HL: SVL, HL: HW, HL: HD, SL: HL, SL: SVL, EN: NS, EN: HL, ED: HL, ED: SL, ED: SVL and ED: EN ratios of both male and female anuran species sampled from unsprayed transplanted rice crop fields were 6.14, 16.74, 6.39, 3.97, 10.76, 32.05, 12.14, 29.96 15.98, 7.74, 34.89 and 2.65, respectively as compared

Table 3. Mean Anuran population (individuals/200m^2) recorded in selected sprayed and unsprayed transplanted rice crop fields of district Ludhiana

Villages	Mean anuran population (2021 & 2022)	Increase in population (%) (2021 to 2022)
Noorpur Bet	10.31	3.85
Ladian Kalan	9.37	13.92
Kothe Sherjang	9.99	5.34
Mean	9.89	-
Research area (School of Organic Farming), PAU	13.59	13.51
Sahibana	12.49	8.07
Burj Lambran	14.30	10.44
Mean	13.46	-

*Mean anuran population values represent the average number of individuals per sampling unit (individuals/200m^2) calculated across three replications during 2021 and 2022

Table 4. Percent difference (between mean body weights of anuran species recorded from sprayed and unsprayed transplanted rice crop fields of district Ludhiana

Anuran species	Mean (2022 over 2021)	
	Male	Female
Duttaphrynus melanostictus	1.72	2.70
D. stomaticus	4.23	1.38
Euphlyctis cyanophlyctis	13.26	12.50
E. adolfi	61.40	41.85
Fejervarya limnocharis	2.96	3.08
Hoplobatrachus tigerinus	1.70	5.55
Mean	14.21	11.17

Table 5. Percentage (%) difference between morphometric parameters (mm) of anuran species from sprayed and unsprayed transplanted rice crop fields of district Ludhiana (2022 over 2021)

Morphometric parameters	*Duttaphrynus melanostictus*		*Duttaphrynus stomaticus*		*Euphlyctis cyanophlyctis*		*Euphlyctis adolfi*		*Fejerverya limnocharis*		*Hoplobatrachus tigerinus*		Mean
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	
SVL	5.99	5.63	5.31	8.52	4.21	5.34	10.83	15.29	4.57	3.15	2.36	2.54	6.14
HL: SVL	21.42	9.02	5.49	12.77	8.45	11.51	21.05	75.00	6.34	14.09	9.13	6.63	16.74
HL: HW	3.53	7.96	3.30	3.98	7.35	12.87	12.38	10.71	2.97	3.48	3.03	5.13	6.39
HL: HD	4.48	4.71	1.62	2.15	6.15	5.12	2.65	10.43	4.25	2.35	1.32	2.44	3.97
SL: HL	6.49	16.00	3.57	8.11	9.92	18.27	16.92	8.82	7.90	16.66	7.64	8.87	10.76
SL: SVL	59.09	35.71	52.00	19.88	26.27	20.26	42.85	8.69	36.60	30.60	37.39	15.33	32.05
EN: NS	5.76	2.85	2.81	6.42	10.97	8.01	4.00	81.81	6.89	7.33	5.08	3.82	12.14
EN: HL	19.86	32.14	45.95	40.23	11.47	21.71	32.17	64.28	20.66	20.19	34.26	16.66	29.96
ED: HL	7.76	15.72	15.22	16.36	21.72	14.57	18.51	30.35	24.16	10.41	11.06	5.98	15.98
ED: SL	6.47	4.95	3.06	4.68	9.13	3.32	6.77	15.20	9.98	3.61	12.09	13.72	7.74
ED: SVL	8.13	18.04	36.00	37.24	54.36	44.27	17.14	100.0	29.41	26.08	34.56	13.55	34.89
ED: EN	2.18	2.88	2.20	3.58	1.21	2.46	3.18	3.70	0.71	3.04	1.88	4.86	2.65

* HL= Head length (from back of mandible to tip of snout) (mm), HW= Head width (left side back of mandible to right side back of mandible) (mm), HD= Head depth at angle of jaw (depth of head at angle of jaw) (mm), SL= Snout length (distance between anterior comer of eye to the tip of the snout) (mm), EN= Eye to nostril distance (distance between anterior point of eye and nostril) (mm), NS= Nostril to snout distance (distance between anterior point of nostril to tip of the snout) (mm) and ED= Eye diameter (mm)

to sprayed fields (Table 5). This indicates that pesticides may exert sub-lethal physiological effects on amphibians, such as metabolic stress, impaired nutrient assimilation, or disruption of endocrine functions, which can negatively influence growth parameters and body condition. The positive correlation was found between anuran population with atmospheric temperature (˚C), relative humidity (%) and rainfall (mm).

Pesticide residues in soil, water and excretory contents: Use of different pesticides by farmers to raise rice crop, the pesticide residues were below the limit of quantification (0.01 mg kg^{-1}) in soil, water and excretory contents (urine and feaces) samples of selected transplanted rice crop fields. The collected amphibians do not show any morphological abnormality due to toxicological effect of pesticide residues which was supported by the absence of any pesticide residues in excretory contents (urine and feaces). From the soil samples, a range of 88.36-108.50%, 76.27-98.61% and 76.15-105.58% were recovered in case of synthetic pyrethroid, organochlorines and organophosphates, respectively. Similarly from the water samples, 76.16-105.40%, 73.71-104.16% and 81.78-107.20%. From the urine samples, percent range of 78.46-101.30, 79.84- 103.71 and 76.11-102.46 were recovered in case of synthetic pyrethroids, organochlorines and organophosphates, respectively. From the faecal matter samples, a range of 79.54-101.27%, 86.43-107.13% and 73.35-107.38% were recovered in case of synthetic pyrethroids, organochlorines and organophosphates, respectively.

CONCLUSION

The study conclude that when rice agroecosystems are combined with nearby natural aquatic and terrestrial habitats, they help to preserve a dynamic, heterogeneous system that acts as a haven for biodiversity. Farmers can improve ecosystem quality to conserve anurans by enhancing judicious usage of pesticides and increasing uncultivated areas like ponds and ditches near the crop fields especially rice crop.

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