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Evaluating acute toxicity of amino acid ionic liquids towards *Poecilia reticulata* fish for designing sustainable chemical processes

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$[N_{4444}][PHE]$
$[N_{4444}][SER]$
$[N_{4444}][GLY]$

ABSTRACT

An acute toxicity study assessed the LC_{50} values for eight different amino acid ionic liquids (AAILs), featuring two cations, tetrabutylphosphonium $[P_{4444}]$ and tetrabutylammonium $[N_{4444}]$, coupled with four anions [PHE], [ASP], [SER], and [GLY]. According to the OECD 203 standard for acute fish toxicity tests with guppy fish (*Poecilia reticulata*, all the AAILs exhibited low toxicity levels, and were practically nontoxic and harmless. The LC_{50} values surpassed 100 mg/L and 1000 mg/L. This study provides valuable insights for industrial professionals in utilizing tetrabutylphosphonium-based amino acid ionic liquids $[P_{4444}]$ [AA] and tetrabutylammonium-based amino acid ionic liquids $[N_{4444}][AA]$ in chemical processes, indicating their safety in aquatic environments. These promising results highlight the potential of incorporating these AAILs into diverse chemical processes while ensuring minimal ecological impact.

1. Introduction

Task-specific ionic liquids (TSILs), also known as functionalized ILs, are salts that have the normal features of an ionic liquid (IL), including functional groups with specific applications that are covalently bonded to cations and/or anions. In this regard, the type of inserted functional group and its location in the structure may also have an impact on the features of TSILs, in addition to the cation-anion combination. TSILs have recently received increased amounts of attention in a wide range of sectors due to their ability to synthesize ILs with specific physicochemical and biological characteristics [1]. TSILs are claimed to be environmentally friendly ILs because of their low vapour pressure, nonflammability [1], thermal stability [1,2], low toxicity [1–3], nonvolatility, low melting temperature, high decomposition temperature, low viscosity [2] and biodegradability [1,3].

Due to the special qualities of ILs, such as their low vapour pressure, thermal and chemical stability, nonflammability, strong ionic conductivity, broad electrochemical potential window, and solvation ability, ILs are gaining increasing attention. An enormous variety of distinct ILs may be created by combining various anion and cation groups or by modifying their properties, such as adding oxygenation groups or changing the length of their alkyl chains. Hence, physical characteristics, including density, hydrophobicity, and viscosity, as well as solubility behaviour and the capacity to degrade biologically or have toxicological effects, can be regulated [4]. ILs are a novel group of chemicals with promising potential as eco-friendly solvents in both industry and academic research for chemical processes. Despite not being widely used in industry, limited information is available about the

Abbreviations: AAILs, amino acid ionic liquids; AAs, amino acids; TSILs, task-specific ionic liquids; ILs, ionic liquids; $[P_{4444}]$, tetrabutylphosphonium; $[N_{4444}]$, tetrabutylammonium; [ASP], aspartate; [PHE], phenylalaninate; [SER], serinate; [GLY], glycinate; $[P_{4444}][AA]$, tetrabutylphosphonium-based amino acid ionic liquids; $[N_{4444}][AA]$, tetrabutylammonium-based amino acid ionic liquids.

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environmental release of these materials [5].

There is a wide range of ILs available, but this research focused particularly on AAILs. Amino acids (AAs) are unique in that they may be readily transformed into both anions and cations, and the diversity of functional groups found in their side chains allows for the easy insertion of chirality and a broad range of functionalities into ILs. Compared to the rest of the chiral pool, AAs are inexpensive and abundant, which allows AAILs to play an important role in green and sustainable chemistry [3] with many advantages. In addition, some AAILs also have disadvantages, such as toxicity. The cation group is mostly responsible for determining the toxicity trend, with the anion having only a minor and supporting function. [6].

On the other hand, aquatic pollution plays an important role in causing a negative impact on all biological species, including humans [7]. Therefore, acute toxicity or ecotoxicology is crucial in assessing the risk of harmful concentrations of ILs to aquatic life. These scenarios are caused by accidental spills, leaching from landfill sites, or industrial discharge [5]. The OECD 203 acute toxicity test is a useful method and provides a preliminary analysis for comparing the effects of various chemicals, including AAILs. Chemicals or AAILs will have direct and immediate impacts on freshwater fish due to their higher trophic level in aquatic food chains. Common freshwater fishes, such as *Poecilia reticulata* (guppy fish), can withstand various kinds of water conditions, making them good choices for ecotoxicology studies.

In this study, two cations, tetrabutylammonium [N_{4444}] and tetrabutylphosphonium [P_{4444}], were paired with four AA anions (aspartate [ASP], phenylalaninate [PHE], serinate [SER], and glycinate [GLY]) to determine their toxicity to guppy fish. The synthesized AAILs were examined to identify the effects of various groups of cations and anions combined with different functional groups and alkyl chain lengths. Therefore, the OECD 203 method was utilized to determine the toxicity of the synthesized AAILs.

2. Materials and methods

2.1. Materials

Tetrabutylphosphonium hydroxide [P_{4444}] [OH] and tetrabutylammonium hydroxide [N_{4444}] [OH] (40 wt%) solutions were purchased from Aldrich. L-Phenylalanine, L-aspartic acid, L-serine and glycine for analysis were purchased from Merck. All the AAILs were prepared and synthesized using a neutralization reaction. The fish species used for this study were only male guppies. The fish used were selected based on the OECD 203 standard and were purchased from an aquatic pet shop in Ipoh, Perak.

2.2. Synthesis of amino acid ionic liquid (AAIL)

The measured amount of AAs salt was diluted in deionized water and stirred at room temperature for 24 hours for complete and uniform dissolution. The [P_{4444}] [OH] solution was reacted with a slight excess of AAs (without further purification) at a ratio of 1:1.05 through neutralization at room temperature for 24 hours. After 24 hours, the ILs underwent evaporation using a rotary vacuum evaporator (temperature: 40–50°C; pressure: 60–80 mbar) to remove water. Then, further purification was carried out using an organic solvent mixture of 10 ml of methanol and 90 ml of acetonitrile. Methanol precipitates unreacted AAs, and acetonitrile lowers the boiling point of the mixture by forming an azeotrope system for the removal of unreacted AAs. The unreacted AAs were filtered out, and the solution was evaporated again to remove the organic solvents.

The AAIL products obtained were dried at 50°C and 500 mbar for 48 hours. All the AAILs were synthesized by using the same procedure. Fig. 1 in reference to [6,8] shows the synthesis route of tetrabutylphosphonium glycinate [P_{4444}] [GLY] using a neutralization reaction. The synthesis route was similar for all the synthesized AAILs in this study. Table 1 shows the list of cations and anions used to synthesize the AAILs in this study.

2.3. Structural characterizations of AAILs

2.3.1. NMR characterization

^{1}HNMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE III Spectrometer operating at 500 MHz with D_2O as the solvent.

2.3.2. FTIR characterization

FTIR spectra were recorded on a Perkin Elmer (Model Frontier 01) in the mid-region of 4000–600 cm ^{1}

2.4. Test organism

The freshwater, male guppy fish used in the test were purchased from an aquatic pet shop located in Ipoh, Perak, Malaysia. The fish were subsequently brought to the laboratory of the Ionic Liquid Research Centre (CORIL) and provided with enough water and oxygen. The fish were kept for 14 days to acclimatize to the new environment before toxicity was evaluated. The length of the fish was approximately 2 cm (the approximate weight was 0.5 g).

2.5. Acute toxicity test

The acute toxicity of the eight ILs toward guppy fish was evaluated in a static environment to determine their fatality after 96 hours of exposure. The fish were acclimated in the laboratory for at least 14 days before the toxicity test. Lake water was utilized instead of tap water for freshwater fish. This procedure was used to avoid chlorine contamination in the tap water. The fish were kept under usual laboratory lighting conditions with a photoperiod of 12–16 hours each day. The water temperature ranged between 23 and 25°C. The water's dissolved oxygen concentration and pH were 5–7 ppm and 7 pH, respectively. Every day, the fish were thoroughly examined for symptoms of sickness, stress, physical damage, and death. Dead and abnormal species were promptly

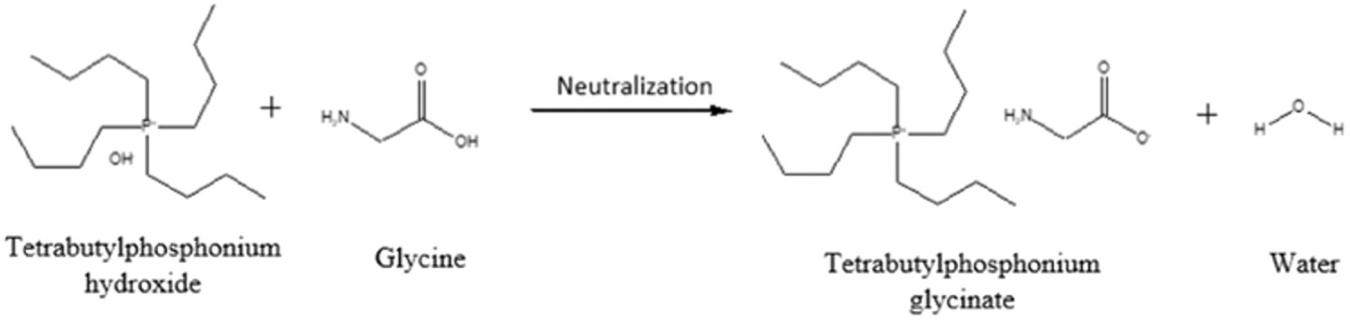

Fig. 1. Synthesis route of tetrabutylphosphonium glycinate [P_{4444}] [GLY] using neutralization reactions.

Table 1
AAIL names and abbreviations used.

CATION	ANION	IONIC LIQUIDS
Tetrabutylphosphonium [P_{4444}]	Aspartate [ASP]	[P_{4444}] [ASP] [N_{4444}] [ASP]
	Phenylalaninate [PHE]	[P_{4444}] [PHE] [N_{4444}] [PHE]
Tetrabutylammonium [N_{4444}]	Serinate [SER]	[P_{4444}] [SER] [N_{4444}] [SER]
	Glycinate [GLY]	[P_{4444}] [GLY] [N_{4444}][GLY]

*4444] and [N_{4444}] cations were paired with four AAs anions: [ASP], [PHE], [SER] and [GLY

Fig. 2. Male guppy fish used in this research.

removed [9].

The fish were kept in the laboratory for 14 days before the toxicity test. For the first week, the fish were fed twice daily, once in the morning and once in the evening [4], and then once a day for the second week. The feeding was then stopped 48 hours before testing [10]. After 48 hours of adaptation, a group of 10 healthy fish was chosen at random and placed in 6.5 L plastic tanks with 5 L of freshwater and electric air pumps for air circulation. The usual swimming manner of healthy fish may be identified. Fish that swim irregularly should not be chosen since they may have a low tolerance to the chemicals used in testing. The fish were not fed anything throughout the experiment. Each fish weighed approximately 0.5 g [6,7,9,11]. The OECD 203 standard procedures [12] were followed for conducting the acute fish toxicity test. At least four concentrations of each IL were examined (0, 25, 50, 75, and 100 ppm). The fish behaviour was thoroughly observed, and any dead fish were removed immediately. After 24, 48, 72, and 96 hours, the number of dead fish at each concentration was counted [6,7,9,11]. The concentration required for the death of 50% of the test fish within 96 hours, or the median lethal concentration (LC_{50}), was subsequently determined. The experimental outcomes were compared to those of the U.S. Fish and Wildlife Service's (USFWS) acute toxicity assessment scale in Table 2 [13]. Theoretical work (PROBIT analysis) (ASTM-E1847, 2008) was conducted based on the observations and findings after 96 hours of the test period [6]. A higher toxicity level is indicated by lower LC_{50} values, which imply that lower concentrations of chemicals result in 50% mortality in organisms.

Table 2
Acute toxicity rating scale by Fish and Wildlife Service (FWS).

Relative toxicity	Aquatic LC_{50} (mg/L)
Super toxic	0.01–0.1
Highly toxic	0.1–1
Moderately toxic	1–10
Slightly toxic	10–100
Practically nontoxic	100–1000
Relatively harmless	>1000

2.6. Statistical analysis

Probit analysis, a specific type of regression model for binomial response variables was employed to establish the LC_{50} value. This involved the observation of fish mortality percentages every 24 hours up to 4 days. Subsequently, the data underwent further analysis through regression to explore the relationship in more detail. The analysis was carried out using SPSS Statistics software (IBM, version 29). The resulting output values, including the 95% confidence interval provide a more precise estimation through the maximum likelihood method [14, 15].

3. Results and discussion

3.1. ^{1}H NMR and ^{13}C NMR characterization of AAILs

Eight AAILs were successfully synthesized and characterized. The ^{1}H NMR and ^{13}C NMR spectra are attached to SUPPLEMENTARY DATA. The attributions of the peaks corresponding to both cations and anions in the ^{1}H NMR and ^{13}C NMR spectra are detailed in Table 3.

The NMR spectra of both [SER] and [GLY] anions exhibited downfield shifts, likely attributed to their compact structures, where the (COO^-), (OH), and (NH_2) groups are closely located. In the ^{1}HNMR spectra, peaks corresponding to (COOH), (OH), and (NH_2) protons could not be detected due to their rapid exchange with deuterium in the solvent, as these protons are highly unstable, causing the original signals to disappear. To confirm the product, a ^{13}C NMR test was conducted, which revealed a particular peak in the range of 177–181 ppm, indicating the presence of (COO^-). This proves the successful synthesis of the AAILs [6,16,17].

3.2. Fourier transform infrared spectroscopy (FTIR)

The FTIR peak attribution for the synthesized AAILs, highlighting the presence of functional groups, is provided in Tables 4 and 5 below. All the FTIR spectra are included in SUPPLEMENTARY DATA for reference.

A notable peak within the range of 1604–1582 cm^{-1} corresponds to the (COO^-) group of the amino acid anion structure. Additionally, the (OH) peak at approximately 3388–3348 cm^{-1}, attributed to water content, overlaps with the N-H peak in the range of 3388–3201 cm^{-1} from the primary amine with (NH_2) incorporated into the anion chain. In addition, all the detailed attributions of specific functional groups for the respective AAILs can be found in Tables 4 and 5. Following the NMR and FTIR results, it was feasible to conclude that the obtained product's molecular characterization was compatible with the AAIL structure and in strong agreement with related findings [16,17].

3.3. Acute toxicity of AAILs toward the guppy fish, Poecilia reticulata

3.3.1. Limit test

Screening tests for the eight AAILs were conducted at a concentration limit of 100 mg/L. Some ILs reveal a toxicity range of 100 mg/L, specifically between 75 and 100 mg/L (where the fish dies). The fish mortality observations for all eight AAILs are detailed in Tables 6 and 7, with each concentration initially comprising 10 fish. The highest guppy mortality occurred at higher concentrations and prolonged exposure, particularly at 100 ppm and 96 hours of testing. The number of dead fish within the prescribed test period was utilized to calculate the mortality percentage, LC_{50} and 95% confidence interval.

3.3.2. Effect on behaviour

Throughout the 96-hour testing period, some fish exhibited distinct apical effects (as stated in OECD 203), such as erratic swimming movements, fin thinning, loss of equilibrium with sideways swimming, sinking to the bottom, and leading to mortality. These manifestations are likely linked to the toxicity impact of AAILs on guppy fish and may be further influenced by the fish's potentially lower resistance or immune levels. On a positive note, there were fish that remained healthy and displayed no adverse effects, thriving for an extended period.

3.3.3. Statistical analysis

A few fish did not survive during the acclimation period, even before exposure to the chemicals. However, no fish died throughout the entire acute toxicity testing period in the control tank. The mortality of guppies for AAIL concentrations of 25, 50, 75, and 100 ppm was examined during exposure times of 24, 48, 72 and 96 hours.

The acute toxicity of eight AAILs with various cations ([P_{4444}] and

Table 3
The cation and anion peaks for ^{1}H NMR and ^{13}C NMR.

ILs	STRUCTURE	^{1}H NMR	^{13}C NMR
[P_{4444}] [ASP]	Cation	^{1}H NMR (500 MHz, D_2O) δ 0.95 – 0.84 (t,12 H; -CH_3), 1.61 – 1.32 (dq,16 H; -CH_2), 2.19 – 2.04 (td,8 H; -CH_2-P^+).	^{13}C NMR (126 MHz, D_2O) δ 12.59 (-CH_3), 17.83, 17.44 (-CH_2-P^+), 22.72, 22.68 (-CH_2-CH_2-P^+), 23.34, 23.21 (-CH_2-CH_3).
	Anion	2.66 – 2.53 (dd,1 H; -CH_2-COOH), 2.80 – 2.66 (dd,1 H; -CH_2-COOH), 3.88 – 3.77 (dd,1 H; -H-NH_2).	36.62 (-CH_2- COOH), 52.29 (-CH-NH_2), 174.10 (-COOH), 177.41(-COO^-),
[P_{4444}] [PHE]	Cation	^{1}H NMR (500 MHz, D_2O) δ 0.92 – 0.86 (t, 12 H; -CH_3), 1.53 – 1.37 (dh,16 H; -CH_2), 2.12 – 2.04 (td, 8 H; -CH_2-P^+).	^{13}C NMR (126 MHz, D_2O) δ 12.66 (-CH_3), 17.86, 17.47 (-CH_2-P^+), 22.76, 22.73 (-CH_2-CH_2-P^+), 23.38, 23.26 (-CH_2-CH_3).
	Anion	2.87 – 2.82 (dd, 1 H; -CH_2-C_6H_5), 3.03 – 2.98 (dd, 1 H; -CH_2-C_6H_5), 3.53 – 3.50 (m, 1 H; -H-NH_2), 7.38 – 7.23 (dt, 5 H; -C_6H_5).	40.73 (-CH_2-C_6H_5), 57.46 (-H-NH_2), 138.35, 129.54,128.67 &126.69 (C_6H_5), 181.77 (-COO^-).
[P_{4444}] [SER]	Cation	^{1}H NMR (500 MHz,) δ 0.97 – 0.84 (t,12 H; -CH_3), 1.60 – 1.36 (ddq,16 H; -CH_2), 2.22 – 2.07 (m, 8 H; -CH_2-P^+)	^{13}C NMR (126 MHz, D_2O) δ 12.61 (-CH_3), 17.87, 17.48 (-CH_2-P^+), 22.75, 22.72 (-CH_2-CH_2-P^+), 23.37, 23.25 (-CH_2-CH_3),
	Anion	3.36 – 3.31 (dd,1 H; -H-NH_2), 3.70 – 3.63 (dd,1 H; -CH_2-OH), 3.77 – 3.71 (dd, 1 H; -CH_2-OH)	57.47 (CH-NH_2), 64.35(CH_2-OH), 179.65 (COO^-).
[P_{4444}] [GLY]	Cation	^{1}H NMR (500 MHz, D_2O) δ 0.95 – 0.84 (t, 12 H; -CH_3), 1.58 – 1.35 (ddq, 16 H; -CH_2), 2.19 – 2.07 (td, 8 H; -CH_2-P^+).	^{13}C NMR (126 MHz, D_2O) δ 12.61 (-CH_3), 17.84, 17.46 (-CH_2-P^+), 22.74, 22.70 (-CH_2-CH_2-P^+), 23.36, 23.24 (-CH_2-CH_3).
	Anion	3.19 – 3.13 (s, 2 H; -CH_2-COO^-).	44.35 (CH_2- COO^-), 180.21 (COO^-).
[N_{4444}] [ASP]	Cation	^{1}H NMR (500 MHz, D_2O) δ 0.92 – 0.77 (t,12 H;-CH_3), 1.33 – 1.19 (h, 8 H; -CH_2-CH_3), 1.62 – 1.47 (p, 8 H; -CH_2-CH_2-N^+),	^{13}C NMR (126 MHz, D_2O) δ 11.99 (-CH_3), 18.25 (-CH_2-CH_2-N^+), 22.23 (-CH_2-CH_3), 57.23 – 57.19, (-CH_2-N^+)

(continued on next page)

Table 3 (*continued*)

ILs	STRUCTURE	^{1}H NMR	^{13}C NMR
		3.17 – 3.02 (m, 8 H; -CH_2-N^+).	
	Anion	2.60 – 2.52 (dd, 1 H; -CH_2-COOH),	35.56 (-H-NH_2), 51.30 (-CH_2-COOH), 173.14 (-COOH),
		2.74 – 2.67 (dd, 1 H; -CH_2-COOH),	176.38 (-COO^-).
		3.80 – 3.75 (dd, 1 H; -H-NH_2).	
[N_{4444}][PHE]	Cation	^{1}H NMR (500 MHz, D_2O) δ	^{13}C NMR (126 MHz, D_2O) δ
		0.97 – 0.89 (t, 12 H; -CH_3),	12.88 (-CH_3),
		1.39 – 1.27 (h, 8 H; -CH_2-CH_3),	19.16 (-CH_2-CH_2-N^+),
		1.65 – 1.54 (dt, 8 H; -CH_2-CH_2-N^+),	23.12 (-CH_2-CH_3),
		3.16 – 3.08 (m, 8 H; -CH_2-N^+).	58.11 – 58.07 (-CH_2-N^+).
	Anion	2.91 – 2.83 (dd, 1 H; -CH_2-C_6H_5),	40.06 (-CH_2-C_6H_5),
		2.91 – 2.83 (dd, 1 H; -CH_2-C_6H_5),	57.21 (-H-NH_2),
		3.58 – 3.52 (m, 1 H; -H-NH_2),	137.81, 129.47,128.71 & 126.83 (C_6H_5),
		7.40 – 7.23 (m, 5 H; C_6H_5).	180.77 (-COO^-),
[N_{4444}][SER]	Cation	^{1}H NMR (500 MHz,) δ	^{13}C NMR (126 MHz, D_2O) δ
		0.99 – 0.88 (t, 12 H; -CH_3),	12.89 (-CH_3),
		1.41 – 1.29 (h, 8 H; -CH_2-CH_3),	19.16 (-CH_2-CH_2-N^+),
		1.70 – 1.57 (p, 8 H; -CH_2-CH_2-N^+),	23.15 (-CH_2-CH_3),
		3.25 – 3.12 (m, 8 H; -CH_2-N^+).	58.15 – 58.11 (-CH_2-N^+).
	Anion	3.40 – 3.35 (dd,1 H; -H-NH_2),	57.40 (CH-NH_2), 64.11(CH_2-OH),
		3.80 – 3.66 (dd,2 H; -CH_2-OH).	179.26 (-COO^-).
[N_{4444}][GLY]	Cation	^{1}H NMR (500 MHz, D_2O) δ	^{13}C NMR (126 MHz, D_2O) δ
		1.00 – 0.87 (t, 12 H; -CH_3),	12.89 (-CH_3),
		1.40 – 1.29 (h, 8 H; -CH_2-CH_3),	19.16 (-CH_2-CH_2-N^+), 23.15 (-CH_2-CH_3),
		1.70 – 1.58 (p, 8 H; -CH_2-CH_2-N^+),	58.15 – 58.11 (-CH_2-N^+).
		3.21 – 3.12 (m, 8 H; -CH_2-N^+).	
	Anion	3.24 – 3.21 (s, 1 H; -CH_2-COO^-)	44.07 (CH_2- COO^-), 179.52 (-COO^-),
		* 1 H is due to high electronegative and deshielding effect	

Table 4
FTIR peak attribution of the synthesized [P_{4444}][AA] ionic liquid.

Functional group	Absorption peak (cm^{-1})			
Absorption peak attribution	**[P_{4444}][ASP]**	**[P_{4444}][PHE]**	**[P_{4444}][SER]**	**[P_{4444}][GLY]**
NH_2 primary amines	3384	3360	3348	3355
OH stretching	3384	3286	3270 & 3201	3279
C-H stretching (alkene)	-	3082	-	-
	-	3061	-	-
	-	3058	-	-
C-H stretching (alkane)	2959	2960	2960	2959
	2932	2932	2934	2932
	2872	2874	2873	2871
CO_2	2247	-	-	-
COO^-	1604	1583	1597	1584
C-H bending	1460	1496	1465	1462
	-	1464	1383	1380
	-	1380	-	-
O-H bending	1369	-	1338	-
	1346	-	-	-
C-N stretching	1229	1237	1236	1238
	1153	1097	1098	1097
	1096	1053	1044	1051
	1047	-	-	-
C═C bending	-	970	-	-
	-	818	-	-
Benzene derivative	-	701	-	-

Table 5
FTIR peak attribution of the synthesized [N_{4444}][AA] ionic liquid.

Functional group	Absorption peak (cm^{-1})			
Absorption peak attribution	**[N_{4444}][ASP]**	**[N_{4444}][PHE]**	**[N_{4444}][SER]**	**[N_{4444}][GLY]**
NH_2 primary amines	3388	3365	3354	3367
OH stretching	3388	3286	3270	3290
C-H stretching (alkene)	-	3084	-	-
	-	3061	-	-
	-	3027	-	-
C-H stretching (alkane)	2961	2933	2959	2960
	2936	2960	2947	2932
	2873	2875	2873	2874
CO_2	2247	-	-	-
COO^-	1603	1596	1590	1582
C-H bending	1461	1490	1463	1462
	-	1465	1382	1380
	-	1382	-	-
O-H bending	1373	-	1336	-
	1348	-	-	-
C-N stretching	1230	1154	1286	1153
	1152	1111	1152	1109
	1109	1033	1039	1031
	1039	-	-	-
C═C bending	-	886	-	-
	-	814	-	-
Benzene derivative	-	701	-	-

[N_{4444}]) paired with four different anions ([PHE], [ASP], [SER], and [GLY]) toward guppy fish was investigated in this work. The 96-hour LC_{50} values for all the AAILs tested were greater than 100 mg/L. According to the Fish and Wildlife Service's (FWS) Acute Toxicity Rating Scale, the results showed "practically nontoxic" values with LC_{50} values greater than 100 mg/L (Table 2). Table 8 displays the results of the limit test on LC_{50} values and 95% confidence limits for all eight AAILs tested.

3.3.4. Discussion

A prior investigation of the toxicity of ILs against zebrafish revealed that the chemical structure of ILs can produce various effects on fish. For example, imidazolium, pyridinium, and pyrrolidinium have LC_{50} values greater than 100 mg/L and may be regarded as nontoxic to zebrafish [6,18]. ILs exhibit various levels of toxicity to aquatic life, ranging from bacteria to fish, with the cationic group being the primary determinant of toxicity. It is strongly influenced by the side chain length. Recently, there has been evidence that anions can contribute to toxicity, but the consequences are usually less severe than side-chain impacts [19,20]. Several forms of anions were used to assess the toxicity of imidazolium and pyridinium-based ILs against algae. These authors discovered that changes in the anionic structure of ILs are considerably less sensitive to algae than differences in alkyl chain length [20].

Previous research has shown that fish are less sensitive to ILs toxicity than are other species at lower trophic hierarchy levels [6,21]. The toxicity of pretilachlor to guppies increases with increasing concentration and exposure time [7]. Certain ILs are potentially hazardous to aquatic life (guppy fish), and the results clearly show that ILs may have completely different effects on guppy fish depending on their chemical

Table 6
Cumulative mortality of Guppy Fish (n=10, each concentration) exposed to [P_{4444}] [AA].

Concentration (ppm)	No. of mortality				Total fish died	Cumulative % mortality
	24 hours	48 hours	72 hours	96 hours		
[P_{4444}] [PHE]						
Control	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0
75	1	0	0	0	1	10
100	0	1	2	2	5	50
[P_{4444}] [ASP]						
Control	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0
75	0	0	0	1	1	10
100	0	1	1	2	4	40
[P_{4444}] [SER]						
Control	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0
75	0	0	0	1	1	10
100	0	0	0	1	1	20
[P_{4444}] [GLY]						
Control	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0
75	0	0	0	0	0	0
100	0	0	0	1	1	10

Table 7
Cumulative mortality of Guppy Fish (n=10, each concentration) exposed to [N_{4444}][AA].

Concentration (ppm)	No. of mortality				Total fish died	Cumulative % mortality
	24 hours	48 hours	72 hours	96 hours		
[N_{4444}] [PHE]						
Control	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0
75	0	0	0	0	0	0
100	0	0	1	2	3	30
[N_{4444}] [ASP]						
Control	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0
75	0	0	0	0	0	0
100	0	0	0	0	0	0
[N_{4444}] [SER]						
Control	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0
75	0	0	0	0	0	0
100	0	0	0	0	0	0
[N_{4444}] [GLY]						
Control	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0
75	0	0	0	0	0	0
100	0	0	0	0	0	0

* n - is the number of fish in each tank. Each tank consists of 10 guppy fishes.

Table 8
Limit test of eight AAILs towards guppy fish.

AAILs	LC_{50} 96 hours (mg/L)	95% Confidence limit (mg/L)
[P_{4444}][PHE]	> 100 [6]	104.87
[P_{4444}][ASP]	> 100	106.47
[P_{4444}][SER]	> 100	191.40
[P_{4444}][GLY]	>1000	1022.38
[N_{4444}][PHE]	> 100 [6]	206.133
[N_{4444}][ASP]	>1000	Toxic effect is not observed
[N_{4444}][SER]	>1000	Toxic effect is not observed
[N_{4444}][GLY]	>1000	Toxic effect is not observed

structure, which is the interaction between the cation and anion. Furthermore, ionic liquids with longer alkyl chain lengths are more lipophilic, which improves membrane permeability. Ionic liquids with longer alkyl chain lengths are absorbed into the lipid bilayer membranes of guppy fishes, changing their ion permeability and eventually killing those fish [20].

In our study, the [P_{4444}] and [N_{4444}] cations coupled with the [PHE] anion led to a practically nontoxic (>100 mg/L) nature towards the guppy fish. This finding agrees with that of reference [6], who carried out studies on the influence of different head groups, functionalized side chains and anions of ILs on aquatic zebrafish. According to the results in Table 8, [P_{4444}][PHE] and [N_{4444}] [PHE] were categorized as nontoxic

due to the benzene ring, which is attached to [PHE] and causes more toxicity to guppy fish than other anions.

Specifically, the mortality rate in $[P_{4444}][AA]$ was greater than that in $[N_{4444}][AA]$. This is due to the higher molecular weight and high reactivity of phosphorus. Phosphorus is a larger and less electronegative atom than nitrogen. A larger amount of phosphorus can contribute to a more polarizable cation, potentially leading to stronger interactions with biological molecules, and may have a greater affinity for lipids, affecting cell membranes more profoundly. This finding agrees with reference [6], which stated that the influence of the head group has little effect on lipophilicity and toxicity. Lipophilicity was used to assess the toxicity of cations, and the most significant factor influencing the change in lipophilicity was the side chain. When polar functional groups were introduced into a short side chain, the reported toxicity was consistently lower than that of the butyl side chain [6].

$[P_{4444}]$ [ASP] is also categorized as practically 'nontoxic', with a total mortality rate of 4 fishes at 100 mg/L because of the carboxylic group (COOH) attached to its alkyl chain, which increases the number of hydrogen bonds and cation-anion interactions. $[P_{4444}][SER]$ also classified as 'nontoxic' with a mortality rate of 2 fishes at 100 mg/L. The hydroxyl group in $[P_{4444}][SER]$ introduces polar interactions, potentially affecting solubility, interactions with biomolecules, and is slightly toxic compared to [P4444][GLY].

A greater interaction leads to greater toxicity. $[P_{4444}][GLY]$, which are categorized as relatively harmless (>1000 mg/L), are associated with fewer interactions between cation anions because both anions lack functional groups, and a simple molecule.

On the other hand, $[N_{4444}]$ coupled with [ASP], [SER] and [GLY] are categorized as relatively harmless (>1000 mg/L) because the mortality rate of guppy fish is zero. This finding agrees with reference [6,18], which highlighted that ammonium salts have a lower LC_{50} than organic solvents and tertiary amines. This difference might be due to the nitrogen atom having a lower molecular weight, high electronegativity and low reactivity. $[N_{4444}]$ generally has lower lipophilicity than $[P_{4444}]$. Because of its lower lipophilicity, $[N_{4444}]$ may interact less with the lipid components of cell membranes in guppy fish, leading to reduced toxicity. $[N_{4444}]$ [AA] has a higher water content, which may also reduce the toxicity level.

In brief, all AAILs are classified as nontoxic or relatively harmless. According to previous reports [6,22], AAILs consisting of 1-(2-hydroxyethyl-3-methylimidazolium) cations paired with glycinate, serinate, alaninate, and prolinate anions were environmentally friendly according to antimicrobial tests of green algae, *Scenedesmus quadricauda* and bioluminescent marine bacteria, such as *Vibrio fischeri,* which are more sensitive than fishes. This proves that the presence of a short alkyl chain and lack of functional groups are ecologically harmless characteristics of AAs anions.

4. Conclusion

In summary, the study established that both $[P_{4444}][AA]$ and $[N_{4444}][AA]$ demonstrate low acute toxicity to guppy fish, as evidenced by LC_{50} values surpassing 100 mg/L and 1000 mg/L, respectively. This indicates their nontoxic nature, suggesting that these materials are safe choices for industrial applications without causing substantial harm to aquatic environments. Furthermore, the findings underscore the potential of AAILs for use in chemical processes with minimal adverse effects. As these ILs exhibit low toxicity, they represent promising alternatives that contribute to environmentally sustainable practices in various industrial applications. However, ongoing research and careful consideration are crucial to ensure the responsible use of these substances and mitigate any potential ecological impact associated with chemical processes utilizing AAILs.

CRediT authorship contribution statement

Noorhafizah Bt Hasanudin: Review only; **Dr Normawati Bt M Yunus:** Review only **Dr Noraini Abd Ghani:** Review & editing, Supervision; **Jivana Parameswaran:** Writing - original draft, Editing, Methodology, Investigation, Formal analysis

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jivana Parameswaran reports financial support was provided by Universiti Teknologi PETRONAS Fundamental and Applied Sciences Department. Jivana Parameswaran reports a relationship with Universiti Teknologi PETRONAS Fundamental and Applied Sciences Department that includes: funding grants. Jivana Parameswaran reports a relationship with Universiti Teknologi PETRONAS Fundamental and Applied Sciences Department that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.03.014.

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