Using Morphology and Genomic Template Stability (GTS) to Track Herbicide Effect on Some Submersed Aquatic Plants

Kun Silprasit[1,*], Arin Ngamniyom[1], Patarapong Kerksakul[1], Sirikul Thumajitsakul[2]

[1] Faculty of Environmental Culture and Ecotourism, Srinakharinwirot University, Bangkok, Thailand
[2] Faculty of Health Science, Srinakharinwirot University, Nakhon Nayok, Thailand
[*] Corresponding author: Email: kun@g.swu.ac.th

Abstract

This study aimed to detect the genotoxic effects of glyphosate on aquatic plants by the RAPD-PCR technique. Native aquatic plants were screened for herbicide testing. The aquatic plant amounts and species were counted and a comparison was made between natural ponds and paddy fields for a total of 17 sites; paddy fields (site 1-10), ponds beside paddy fields (site 11-14), and natural ponds (site 15-17). At each studied site, 5 randomized sampling plots of 2 x 5 m size were performed. Generally, the natural ponds contained more aquatic plant diversity than the paddy fields. However, some species such as *Najas graminea* Del. and *Ceratophyllum demersum* Linn. were found only in the paddy fields and natural ponds, respectively. The effect of glyphosate and butachlor on aquatic plants was observed. Changes in color and morphology were found to be related to higher dose treatment. The RAPD profiles were analyzed for the study of genomic template stability (GTS). Results indicated that the GTS of *Hydrilla verticellata* was lowest (7.14 %GTS), followed by *Utricularia aurea* lour (30.77 %GTS), *N. graminea* Del. (38.71 %GTS), and *Nitella* sp. (59.38 %GTS). These results confirmed the effects of herbicides on abnormal morphology and DNA instability. In addition, results of genotoxicity of glyphosate on some aquatic plants (*Hydrilla verticellata*, *Utricularia aurea* lour, and *N. graminea* Del.), and macroalgae (*Nitella* sp.) verified that the method of RAPD-PCR could be used as a biomarker to detect herbicide contamination in aquatic ecological systems.

Keywords: Genomic template stability; Aquatic plant; Glyphosate; Butachlor

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Introduction

Human activities such as the utilization of herbicides and insecticides could result in dramatical changes in natural aquatic ecosystems [1]. In Thailand, most agricultural land is used for rice cultivation and there was an increase in the use of herbicides such as glyphosate and butachlor of over 60 million liters during 1998 and 1999 [2, 3]. As most of herbicides are not easily degraded, large amounts of the residues could be remained and cause negative impacts to both non-target organisms and surrounding environment [4]. Some herbicides such as Roundup, Stomp, and Reglone can affect the DNA structure in non-targeted living organisms [5], and cause genotoxic effects on *Biomphalaria glabrata* snails [6]. Dimitrov et al. (2007) studied the genotoxicities of the herbicides glyphosate, pendimethaline, and diquat on *Crepis capillaris* L. and found the increase in micronucleus frequency in the plant cells which might involve the spindle-destroying effect [7]. In the case of genotoxicity, several bioassays such as chromosome/chromatid aberrations and micronuclei can be used to monitor effects in organisms [8]. Plants such as *Allium cepa*, *Vicia faba*, *Trifolium repens*, and *Tradescantia virginiana* were used as bioindicators of genetic toxicity and mutagenic activity of the environmental pollutants [9]. In some cases, chromosome aberration assays, mutation assays, cytogenetic tests, and specific locus mutation assays were performed on plants to detect the heavy metal damage.

The advantage of measuring the effect of genotoxic chemicals directly on DNA is mainly related to sensitivity and short response time. The random amplified polymorphic DNA-PCR (RAPD-PCR) technique can be used as a powerful tool to detect induced genotoxicities by toxic agents in the aquatic system [10-13]. In this study, local submersed aquatic plants were selected from paddy fields, ponds beside paddy fields, and natural ponds. The effect of herbicide on these plants was studied by varying the dose of the treatment. The PCR method was used to investigate DNA stability. The objectives were to detect the genotoxicity effects of glyphosate on some aquatic plants (*Hydrilla verticellata*, *Utricularia aurea* lour, and *Najas graminea* Del.), and macroalgae (*Nitella* sp.) by RAPD-PCR technique.

Method
1) Study areas

Nakhon Nayok Province is situated in the central eastern part of Thailand. Approximately 50 % of the land is used for agriculture (1,292 sq.km) and of that, 80 % is paddy fields with pesticide treatment performed every crop season. About 70% of the Ongkharak District, the study areas, are paddy fields (Figure 1). The study areas were selected according to long-term herbicide treatment during the crop season. In addition, a double-crop field or off-season rice cultivation was usually performed in these areas.

Prior to rice cultivation, paddy fields were usually prepared using the glyphosate herbicide for weed elimination, and the fields were then rested for 7-10 days before sowing the rice seeds. After rice germination, butachlor, was used to control other weed growth in the paddy field. Rice was basically grown for 90-120 days before harvesting started [14]. Though, the half-life for glyphosate is 45-60 days, it could remain stable in the soil for 170 days [15]. While the half-life of butachlor is about 19 days [16]. As a consequence, there is a possibility of glyphosate to remain in the soil and contaminate the ecosystem through runoff and drainage. The glyphosate and butachlor treated paddy fields and ponds beside the paddy fields were selected as sampling sites. Some natural ponds were also selected as a control.

Figure 1 Sampling sites for the study of aquatic plant diversity. Sites 1-10, 11-14, and 15-17 were paddy fields, ponds beside paddy fields, and natural ponds, respectively. Size of circles indicating numbers of aquatic plant samples studied.

2) Aquatic plant samples

Aquatic plants collected from all sampling sites were *N. graminea* Del., *Ceratophyllum demersum* Linn., *H. verticellata*, and *Nitella* sp. These four aquatic plant species were selected since they are native plants in the study areas. Moreover, they could grow under similar condition. After collection, test species were grown at room temperature with an alternate 12/12 light-dark cycle using cool-white fluorescent lamps. Plants were grown in freshwater for 30 days to obtain plant samples before bioassays. At the same time of sample collection, diversity study was also conducted by an investigation of plant species and numbers in a 2 x 5 m plot with randomization.

3) Treatment preparation
3.1) Chemicals

Active gradient butachlor is generally used at an application rate of 1.5 mg/L, at 6 L/ha as a pre-emergence herbicide in transplanted paddy.

The SAM, commercial butachlor product, contains about 60% w/v of butachlor as an active ingredient. The commercial product of glyphosate usually contains 48% w/v of glyphosate as an active ingredient. About 2-5 times higher in both herbicide concentrations than the normal application rate (1.5, 3.0, 6.0, and 12.0 mg/L) were used in this study.

3.2) Herbicide treatment

The glyphosate and butachlor solutions were diluted in 50% ethanol. These solutions were then added to each plant growth chamber (20 x 20 x 10 cm clear plastic box) containing water to provide the nominal herbicide concentrations indicated earlier. The final concentration of ethanol was 0.1% and untreated controls received only 0.1% ethanol in water. For short-term experiment, aquatic plants were grown in treated water for 1 month, and morphology such as color, abnormality, and budding was observed. The apical meristem of new budding was col-

lected for genetic stability by PCR method. The long-term experiment was conducted by growing the plant samples over a period of 2 months.

4) Genetic stability tests by PCR method

Genomic DNA was isolated from 5 plant leaves using RBC Real Genomics™ DNA/ RNA Purification kit (Taipei County, Taiwan). The DNA amplification was carried out by PCR technique using 10 standard decamer oligonucleotide primers including C1 ACGGGCGCCA, C2 ACGGGCCCCA, C3 ACGGCCGC CA, C4 ACGGCCCCCA, C5 ACGCGCGC GA, C6 CCAGGCCAGG, C7 GCAGGCCA CG, C8 GCAGCGCACG, C9 GCCAGCAC CG, and C1 GGGAGCAGGG. The volume of 20 µL of total reaction mixture consitsted of 25 ng template DNA, 10 µL of GoTaq® Green Master Mix (GoTaq® DNA Polymerase supplied in 2X Green GoTaq® Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, and 3mM $MgCl_2$) supplied from Promega Corporation (USA) and 4 µL of 5 µM primer was used. The reaction was carried out in a thermal cycle. The pre-denaturation with 95°C for 5 min, following by 35 cycles of denaturation at 95°C for 45 min, primer annealing at 45°C for 45 s, and primer extension at 72°C for 1 min, and then, the last extension at 72°C for 7 min. The PCR products were determined on 1.5 % agarose gel by electrophoresis technique and visualized under UV light. The HAT-RAPD reactions were repeated twice.

5) Estimation of genomic template stability

Analysis of DNA variation was determined by scoring present (1) or absent (0) of a HAT-RAPD band. Polymorphism observed in the DNA profiles included the disappearance of a normal band and the appearance of a new band in treated sample when compared with non-treated sample profiles (set to 100 %) [17]. The GTS was calculated using Eq.1.

$$GTS (\%) = (1-a/n) \times 100 \qquad Eq.1$$

Where;

a is the number of polymorphic bands detected in each treated sample which is equal to the sum of disappearance of a normal band and appearance of a new band

n is the number of total bands in the control

Results and discussion

1) Area description, aquatic plant diversity and water quality

Results of water quality (pH, color, TDS, and turbidity) analyses of all sampling sites were found to be in the same magnitude. The pH of 7.5-6.5, color of 200-500 PCU, TDS of 48-130 mg/mL, and turbidity of 35-260 ntu were determined.

Results of water quality analyses also confirmed that all sampling sites (Figure 1) were in the similar group of water quality habitat classification. Results of aquatic plant diversity studied in the paddy fields (site 1-10), ponds beside paddy fields (site 11-14), and natural ponds (site 15-17) as shown in Figure 2 confirmed the biodiversity of freshwater aquatic plants in the study areas. In short, the diversity varied relating to the water resources. Low numbers of species were found in paddy fields with more numbers observed in natural ponds located away from the paddy fields. The species were in the family Hydrocharitaceae (*N. graminea* Del. and *H. verticellata*), Ceratophyllaceae (*C. demersum* Linn.), Characeae (*Nitella* sp.), and Nymphaeaceae (*U. aurea* lour). Three aquatic plant species (*U. aurea* lour, *N. graminea* Del., and *Nitella* sp) with the dominant of *U. Aurea* were found in the paddy fields. In the case of ponds beside paddy fields, 2 plant species including *U. aurea* lour and *H. verticellata* as dominant were observed. On the other hand, 4 species with the same distribution of *H. verticellata, C. demersum* Linn., *U. aurea* lour, and *Nitella* sp were

determined in the natural ponds. In relation to the water quality, nearly all the aquatic plants were often found in the paddy fields and natural ponds, with very few species found in paddy fields and ponds beside the paddy fields. Some species were found only in paddy field e.g. *N. graminea* Del., while, *C. demersum* Linn. was found in natural ponds. As *H. verticellata* and *U. aurea* lour were found in a wide range of water resources (such as clear to turbid water) and they have a wide range of herbicide tolerance. It was reported by Steinrücken et al. [19] that both *H. verticellata* and *U. aurea* could tolerate to herbicides. In addition, glyphosate and butachlor could cause inhibition effects on aquatic plants like weeds [19]. Thus, these 2 plants could not be used as biomonitoring indices of herbicides. In contrast, some plant species such as *graminea* Del. or *C. demersum* Linn. could be used to indicate the negative impacts of herbicides as they were only found paddy fields and natural ponds, respectively.

2) Aquatic plant morphology affected by herbicide

Herbicide used during rice cultivation can be eventually released and adversely affect aquatic organisms via rain precipitation and surface runoff. In particular, glyphosate is used as a post-emergence herbicide against both broadleaf and cereal weeds to inhibit an enzyme involved in the synthesis of the aromatic amino acids tyrosine, tryptophan, and phenylalanine. Glyphosate is absorbed through the foliage and translocated to the growing points. Because of this mode of action, it is only effective on actively growing plants [19]. Glyphosate generally blocks the activity of the enzyme enolpyruvylshikimate-3-phosphate synthase (EPSPS), the first enzyme of the shikimic acid pathway, and resulted in the significant decrease in chlorophyll a and b contents, photosynthetic pigments as well as the net photosynthetic rate. It should be noted that EPSPS is normally located within the chloroplasts.

Therefore, shed light of plants could be observed after glyphosate exposure. In addition, plants may become deficient in aromatic amino acids, eventually leading to death by starvation after glyphosate exposure as well [21]. While, butachlor is absorbed primarily through the germinating shoots and secondarily by the roots. It is, then, translocated throughout the plant, with higher concentration in the vegetative parts than the reproductive parts, and it is a protein synthesis inhibitor. Butachlor was reported to have approximately 19 days of half-life in soil and 1.65-2.48 days in field water [20]. Therefore, morphological effects are expected to be developed by plants in response to this herbicide.

Results of herbicide treated experiment showed leaf color changed (Figure 3). Butachlor treatment with 1.5 mg/L water on *C. demersum* Linn., *Nitella* sp. and *U. aurea* lour resulted in brown color throughout the whole leaf. While, *N. graminea* Del. showed normal green with yellow leaf color at 3.0 mg/L water. At 6.0 mg/L water, *H. verticellata* and *N. graminea* Del. leaves were light brown. However, morphological effects could not be observed in *N. graminea* Del., *Nitella* sp., and *U. aurea* lour treated with glyphosate. Yellow leaves and light-heavy brown colors could be observed in *H. verticellata* trated with 1.5 mg/L and higher glyphosate concentration, respectively. In the case of *C. demersum* Linn., normal green leaf was obsereved with the treatment of maximum 6.0 mg/L. With higher concentration of glyphosate, leaves of *C. demersum* Linn. changed into dark green. Therefore, it could be concluded that butachlor had more effect on aquatic plants than glyphosate, however, herbicide contamination with high dose caused aquatic plant death.

After long-time treatment with 3.0 mg/L glyphosate, experimental results confirmed the effect of glyphosate on aquatic plant growth. Glyphosate-treated aquatic plants showed darker green color comparing to the untreated experiment. In addition, an abnormal budding as shown

in Figure 4 was also observed. *H. verticellata* showed slow budding growth and the apical meristem became pale, similar to *U. aurea* lour and *N. graminea* Del. *Nitella* sp. apical budding was different from untreated plants, and short shoots were found in treated plants. The diffe-rences in morphologies of treated and untreated aquatic plants strongly confirmed the glyphosate effect on aquatic plant morphology. These dif-ferences can be used as bioindicators of glypho-sate contamination in ponds.
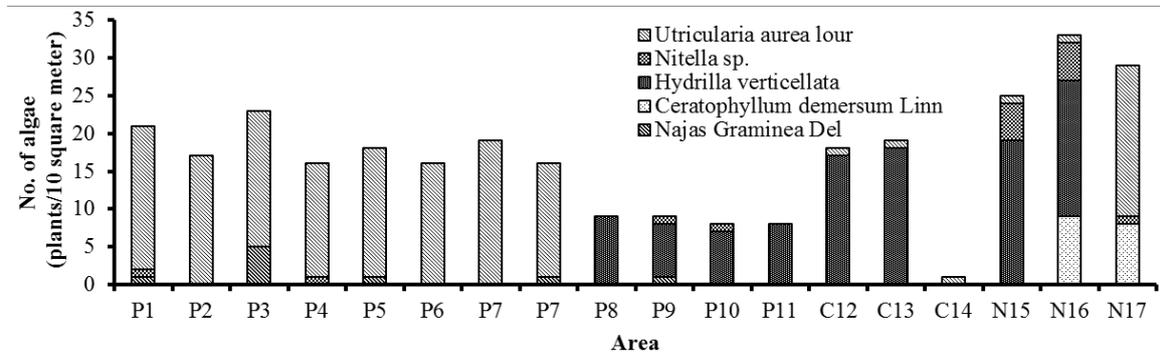

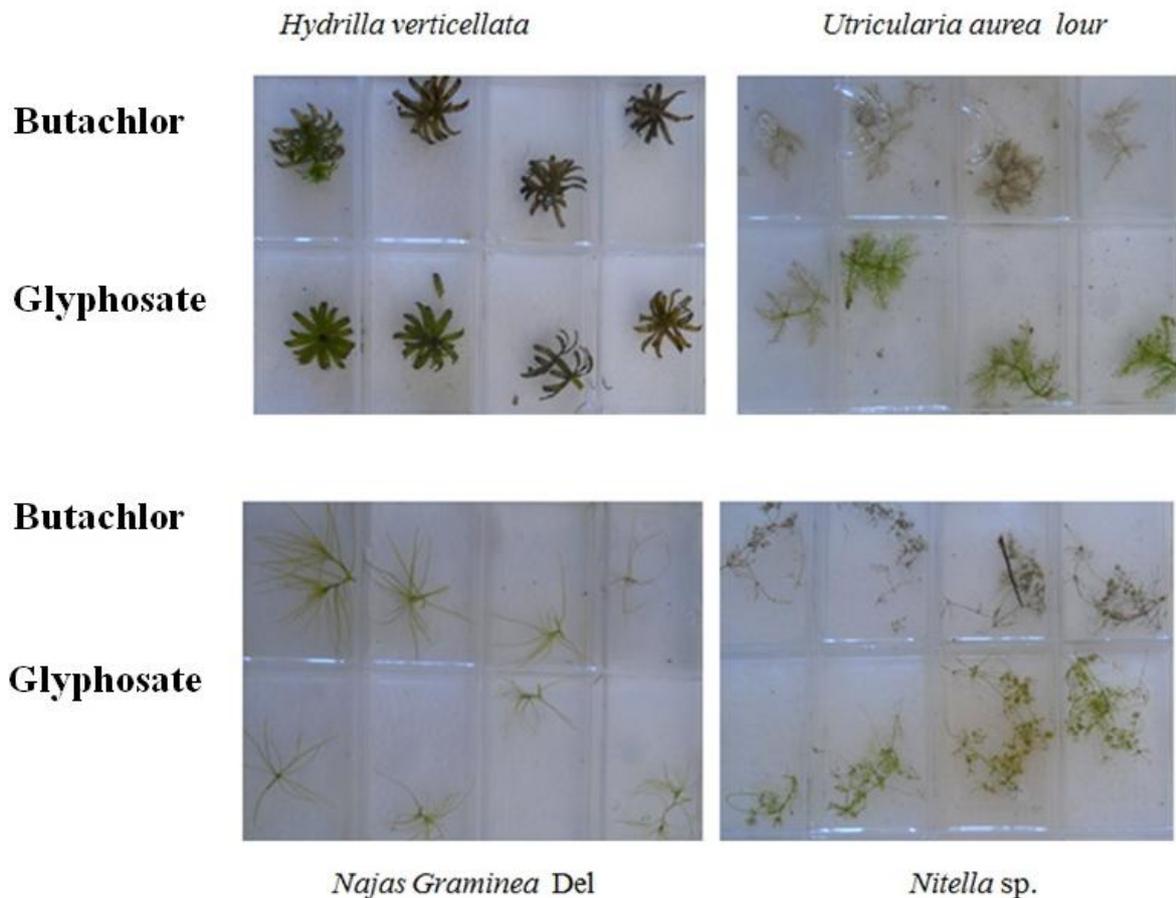**Figure 2** Results of aquatic plant diversity study.

Figure 3 Pictures showing morphological effects of butachlor and glyphosate to aquatic plants. High doses of butachlor caused lethal effects. In contrast to glyphosate effects, plants were still alive after high dose exposure.
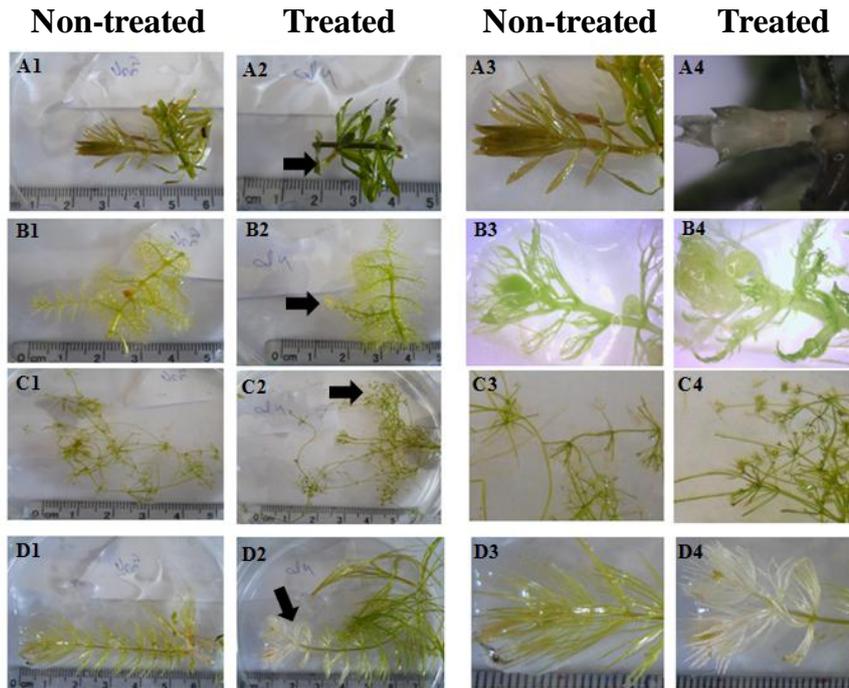
Non-treated   **Treated**   **Non-treated**   **Treated**

Figure 4 Pictures incidating effects of 3 mg/L glyphosate on aquatic plant morphology after 2 months experiment. The details of each capital alphabet were; A for *Hydrilla verticellata*, B for *Utricularia aurea* lour, C for *Nitella* sp. and D for *Najas graminea* Del. In addition, number 1 to 4 indicated the untreated, treated, zoom in untreated and zoom in treated, respectively. A black arrow indicates a point of budding.

3) RAPD indicating herbicide effect

The RAPD bands were scored for genomic template stability (GTS) on the basis of novel biomarker assays for detection of DNA damage and mutations in the cells of bacteria, plants, and animals [22]. The change in the DNA band patterns was expressed as a decrease in GTS. The DNA band patterns for GTS evaluation in *N. graminea* Del., *H. verticellata*, *C. demersum* Linn., *Nitella* sp. and *U. aurea* lour treated with 3.0 mg/L Glyphosate showed DNA damage (Figure 5). Out of 10 primers tested, only 3 gave clear and reproducible bands (Table 1), showing the difference between the non-treated and glyphosate-treated sample. The sizes of DNA fragments were between 100-2000 bp, and polymorphic bands included the appearance of new DNA bands and disappearance of normal DNA bands. The high polymorphic profiles indicated high DNA damage by glyphosate treatment on *H. ver-*

ticellata. The genomic template stability (GTS) was calculated by DNA band patterns from each treated-sample. The lowest GTS was found in *H. verticellata* (7.14% GTS). The GTS level in other aquatic plants were 30.77% GTS for *U. au- rea* lour, 38.71% GTS for *N. graminea* Del. and 59.38% GTS for *Nitella* sp. These results confirmed the effect of DNA damage caused by these herbicides. There were many reports on a significant genotoxic effect especially oxidetive stress of glyphosate on aquatic plants [23-27]. The genotoxic effect of glyphosate involved increasing in the oxidative stress as a secondary effect of the blocked shikimate pathway. Maize leaves exposed to glyphosate showed an increased level of lipid peroxidation, glutathione (GSH), free proline content, and ion flux. In a gene expression analysis, the glyphosate application generates hydrogen peroxide (H_2O_2), resulting in peroxidation and destruction of li-

pids in rice (*Oryza sativa*) leaves. A previous study also reported the increase in genetic damage of plant after 6 days exposure of glyphosate [12]. In addition, the random amplified polymorphic DNA (RAPD) assay was evaluated as a potential tool to detect the ecotoxicity induced by nitrofurazone in the marine ciliate, *Euplotes vannus* [28]. The alteration of DNA structure was investigated in other plants, and induction of chromosomal aberrations was observed in *A. Cepa* root meristem cells and an increase in reverse mutations in *Salmonella typhimurium* TA 98 and TA 100 with glyphosate induced DNA damage in *Rana catesbeiana* tadpoles [29].

Table 1 Changes of total bands in non-treated, polymorphic bands, varied band by primers set and % GTS in aquatic plants

Primer	*Hydrilla verticellata*			*Utricularia aurea* lour			*Najas Graminea* Del			*Nitella* sp.		
	n	**p**	**d**	**n**	**p**	**d**	**n**	**p**	**d**	**n**	**p**	**d**
C1	7	7	7	10	9	3	9	4	5	11	2	2
C4	9	4	1	9	2	1	10	2	1	9	2	3
C7	12	4	3	7	0	3	12	3	4	12	1	3
Total	28	15	11	26	11	7	31	9	10	32	5	8
a			26			18			19			13
a/n			0.93			0.69			0.61			0.41
1-(a/n)			0.07			0.31			0.39			0.59
% GTS			7.14			30.77			38.71			59.38

Note: n is non-treat sample

p is appearance band in treated-sample

d is disappearance band in treated-sample

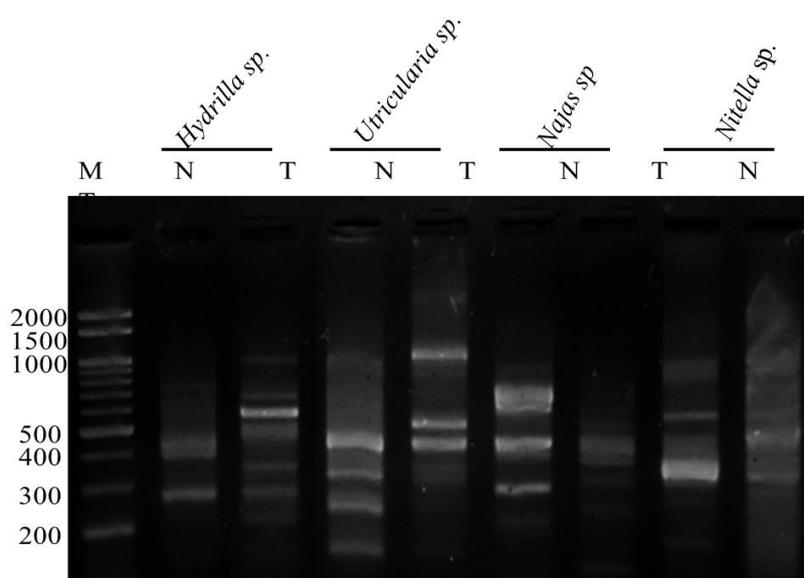a is polymorphic profile (a = d+p)

Figure 5 RAPD profile generated by C1 primer. The profile was compared between non-treated (N) and Glyphosate-treated samples (T) in four aquatic plants species. The polymorphism was determined, including the appearance of new DNA bands and the disappearance of non-treated DNA bands.

Conclusions

The contamination analysis in aquatic systems was found to be difficult because the dose of contamination was diluted with natural phenomena such as precipitation, flooding, as well as biodegradation process. However, biological damage was observed in organisms both animals and plants. This study showed that some herbicides caused untargeted aquatic plant death and reduction in aquatic plant diversity. Butachlor had a greater effect on aquatic plants with the changed in leaf color from green to brown. The plants observed including *N. graminea* Del., *Nitella* sp., *U. aurea* lour, and *H. verticellata* were found to be remained alive in glyphosate-contaminated water. However, the genomic template stability in *H. verticellata* decreased with higher dose of glyphosate treatment. The genetic damage caused by herbicides could be used as a biomarker for detecting herbicide contamination in aquatic ecosystems.

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