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Early detection of lead stress on *Marsilea Crenata* using biolectricity measurement

Arry Y Nurhayati[a]* ,Yuda C Hariadi[a], P Lestari[a]

[a]*Physics Department Faculty of Mathematics and Natural Sciences University of Jember, Jember 68121, East Java Indonesia*

Abstract

Demands on an early detection method for lead (Pb), found as a pollutant in soil, are urgently needed to improve awareness on the continuing usage of waste water and industrial effluent for the agricultural practices especially in East Java, Indonesia. *Marsilea crenata* plant grown in a glasshouse under various concentration of Pb to indicate its tolerance to Pb stress is measured by its weekly biolectrical responses. The result shows that increasing Pb concentration tends to inhibit the plant's growth. It is concluded that the method is viable as an early detection remediation process to protect agricultural economy and human security.

*Keywords:*lead (Pb) stress; Marsilea crenata; biolecetricity; early detection

Nomenclature

Pb	symbol of lead (Plumbum)
PD	Potential difference
DNA	Deoxyribo nucleid acid
S.e	Standart error
LED	Light emitting diode
WHO	World health organization

* Corresponding author. Tel.: +62-81234-65113; fax: 62-331-330-225.
*E-mail address:*arrynurhayati@gmail.com

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1. Introduction

Lead (Pb) polluted soil has been indicated to be one of the main environmental threats in developing countries[1]. Awareness should be improved in Indonesia due to continuing agricultural practice of using waste water and industrial effluent to irrigate plants, especially in East Java. It is evidenced by the detrimental effect of lead in crops grown in polluted soil[2,3,4] which may damage human health through the food chain[5]. Removing lead from the environment is urgently needed and would greatly benefit for agricultural economy and human safety.

Other Pb removal methods have been offered; e.g biosorption and phytoremediation[5, 6], but complete control and prevention over lead exposure are yet to be achieved[1]. The research is aimed at the usage of *Marsilea crenata*as an early detectionfor remediation process by means of detecting its tolerance to Pb stress and observing its growth in a given time.The detection method uses biolectrical responses from the plant[7]. It willalso help to cut costs and fill the gap in environmental remediation process, which in turn saves agricultural economy and protects human security.

2. The Experiment Methods

Seedlings from *Marsilea crenata*[8]were collected from fields during the rice growing season and were then grown in pots containing soil nutrients and water tomaintain the plant's health. The healthy and uniform seedlings were then transplanted into plastic pots containing sterilized sand and a modified Hoagland solution.The each plant was separately exposed to a 0ppm, 3ppm, 10ppm, 20ppm and 50ppm of Pb (as PbO) and grown hydroponically in glasshouses under randomized design, and each was replicated five times for statistical purposes. Sample leaves were taken after 5 weeks of Pb application, then measured by a length x width x factor correction.

The electrical potential differences (PD) on the leave's surface was measured weekly with an electrometer in a Faraday cage. LED lighting was used as artificial sunlight to maintain photosynthesis. Borosilicate glass capillaries, GC 150-10, Harvard apparatus Ltd, UK diameter 1.5 mm (outer) and 0.86 mm (inner) were used to make 10μm diameter tips for the Wick electrodes. The electrodes were filled each with a silver wire containing 1% agar of 1MKCl. Reference electrodes were made using a 5 cm long 2mm diameter plastic tube filled with a silver wire as in[7]. The obtained data was then processed using ANOVA, the significance was accepted at $p<0.05$.

3. Result and Discussion

3.1. Lead Stress of Marsilea Crenata

Inhibited growth from lead toxicity on three most important cerelia crops had been observed in rice[9, 4], maize[10], and wheat[2]. Growth reduction had also been observed in other crops such as mungbean[11]and cowpea[12]. It appeared that lead toxication caused stress on most plants shown by reduced growth such as shoot in ferns[6], reduced leaf blade and changes to stoma and trichomes distribution in cucumber seedling's after 16 days of incubation[13].

Fig.1. (a) *Marsilea crenata* (Semanggi air) grown at Hoagland solution; (b) Pb stress symptoms of *Marsilea crenata*at 7 weeks after initial exposure.

After observing through eight consecutive weeks, the*Marsilea crenata* had shown increasing Pb concentration through reduced growth oftheplants in all treatments by reducing leaf area after the 6^{th} week of application of Pb (Table 1). No visual changes have been observable in the leaves of the plant during the 4 weeks of experiment. The symptoms of lead stress in *Marsilea crenata* was noticed after the 7^{th}week oflead exposure; such as chlorosis on the leaves; dark colouring of stalks, leaves or stem; staining from green (Fig.1a)to a brownish color (Fig.1b); stalk or stem damage, weakened and drooped plant. Brown stalk or stem colour may be caused by increasing concentrations of lead within the stalk or stem due to the increased Pb application concentrations. Early signs of chlorosis were observed on week 6 under 10ppm and 20ppm, but similar trend did not show at 50ppm (Table 2), and after the 8^{th}chlorosis was present on all levels of Pb.

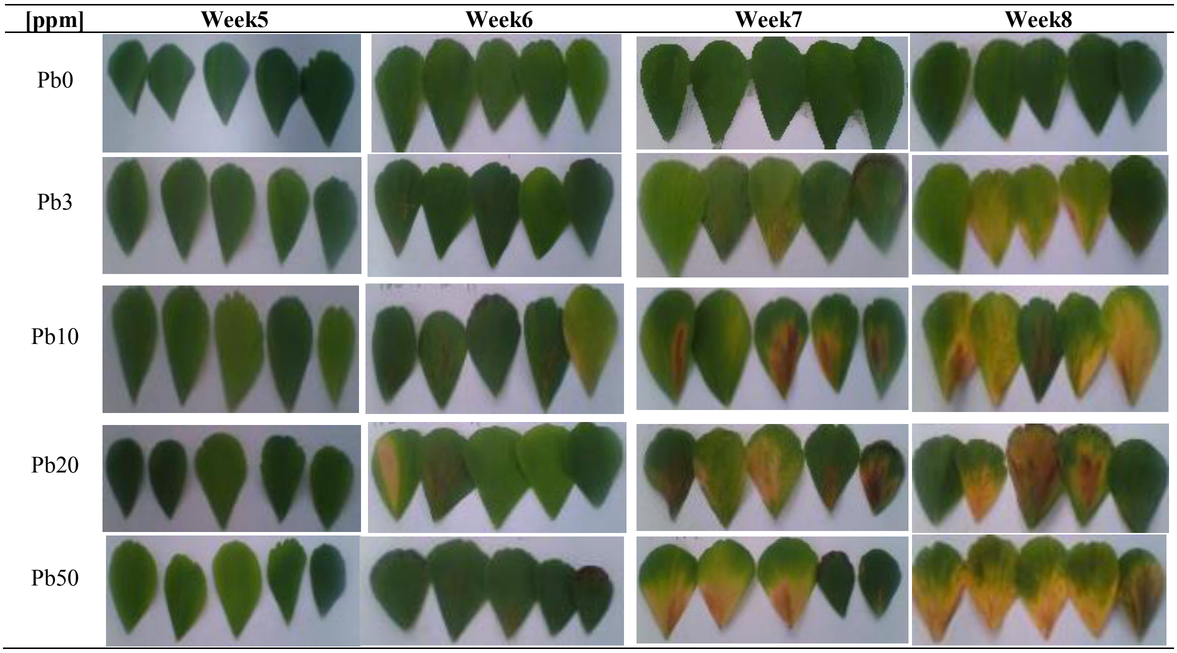
Leafchlorosis, browning of stalks and stem; stunted leaf growth were common symptoms for many plant nutrition deficiencies[14] such as Mg or Fe. Mg deficiency observed on cowpea due to absorption of lead concentrations at Pb ≥ 0.64 µM[12] caused chlorosis in young leaves and growth reduction, while signal grass leaves has shown an interveinal chlorosis in solutions containing 1.5µM of Pb. The severity of chlorosis increased with the amount of concentrations[15]. The chlorosison *Marsilea crenata* might exist due Mg substitution in leaves by Pb. Toxicity symptoms of Pbwere shown by discoloration, pigmentation, yellowing and stunting in leaves which were assessed by eye observation through-out the experiment on six weeks after application of ≥20ppm of Pb and seven weeks after application of ≥ 10ppm of Pb (Table 2).

Table 1.Effect various concentration of lead [Pb] on average of total leaves area of *Marsilea crenata*, and standart error (s.e)for n =5

Week	**Leaf area ± S. e.** [cm^2]				
	[0 ppm]Pb	[3 ppm]Pb	[10 ppm] Pb	[20 ppm]Pb]	50 ppm]Pb
week-5	8.49 ± 0.85	8.75 ± 0.85	8.33 ± 0.56	6.92 ± 0.82	8.04 ± 0.89
week-6	10.46 ± 0.97	8.23 ± 0.74	8.81 ± 0.62	8.57 ± 0.68	8.53 ± 0.67
week-7	12.83 ± 0.61	8.36 ± 0.86	7.84 ± 0.64	8.80 ± 0.87	8.27 ± 0.84
week-8	11.69 ± 0.70	10.37 ± 0.48	9.46 ± 0.76	8.93 ± 0.50	7.76 ± 0.80

The result shown that exposure to Pb in long durations (eight weeks) may directly or undirectly affect the physiological process of *Marsilea crenata* plant (Table2), even at low Pb concentrations levels (3 ppm). The measurement of leaves area supported the visual result that in general increasing Pb concentration, decreases the growth of *Marsilea crenata* by reducing the leaves area of the plant. But the results also shown that the effect of reduction in the leaves area had not shown a concentration dependency during the early weeks, except for week-8 (Table 1, Fig 2).

Table 2.Visual change on leaves of *Marsilea crenata* on grown in the variation of concentration Pb during week five to eight



The result had shown little difference between control and treatment of 3ppm and 10ppm of Pb on *Marsilea crenata* on week-5, while on the 20ppm treatment, average leaf area seemed lower than other concentrations. In general our results had shown that increasing Pb reduces growth of semi aquatic fern *Marsilea crenata*. Our results supported the results in *Hordeum vulgare L* and *Brassica napus L*[16], and in aquatic fern (Azolla)[6].

3.2. Early Detection of Lead Stress on Marsilea crenata using Biolectricity Measurement

Our resultsimplied that chlorosis on *Marsilea crenata* related to exposure of lead greater than 20ppm, was present in a relative period of time. Measuring *Marsilea crenata* growth using biolectricity technique has shown electrical PD of leaves surface (Table 3, Fig.3).It wasaffected afterbeing exposed to various concentrations of Pb. The average leaves surface PD had increased for plants grown without Pb from week 5 to week8, while plants under various concentration of Pb (3, 10, 20 and 50)ppm tended to reduce weekly PD.

The tendency of reduced electrical PD on every treatment (Fig.3) indicated a disturbance in ion transporting process due to Pb application. Destruction of ion transportation might have been caused by the existence of Pb in the growth media of*Marsilea crenata*, and had been caused by the needed mineral nutrition imbalance of the plant. Declining quality of media due to Pb presence might have reduced the root system's function to uptake minerals and nutrients, and in turn had disturbed photosynthesis in *Marsilea crenata*.

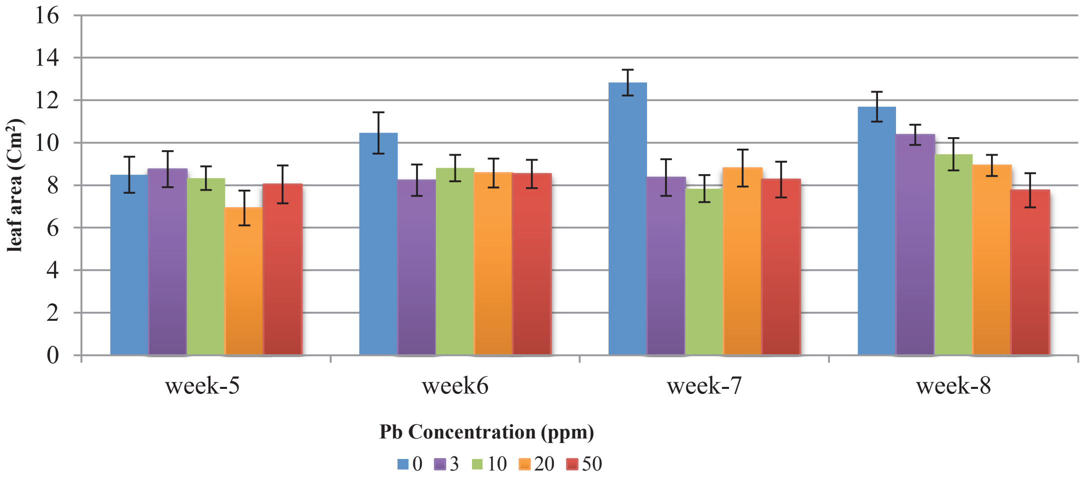


Fig.2. Effect various Pb on the leaf area of *Marsilea crenata* (Semanggi air)

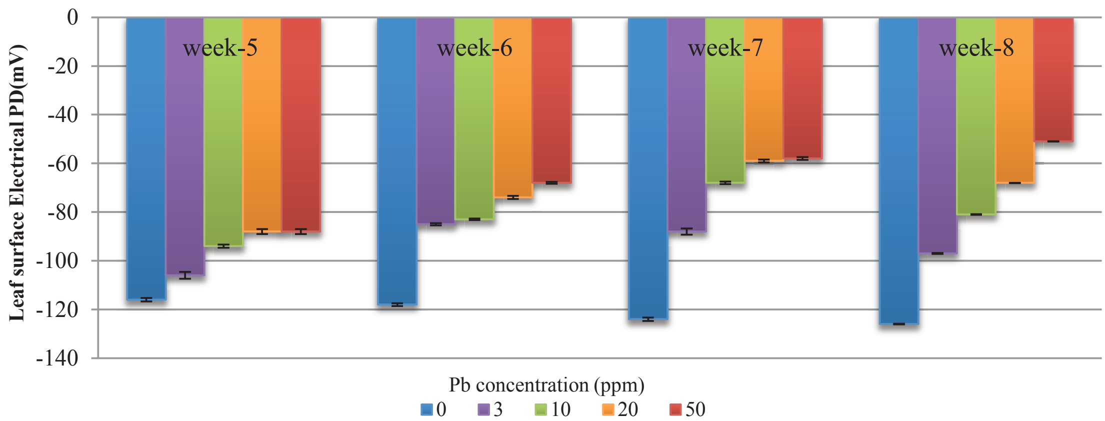


Fig.3.Effect various Pb on leaf potential difference of *Marsilea crenata*. A tendention of reducing PD in earlier week due to stress Pb

Table 3.Leaves surface electrical potential difference (PD) and standard error (s.e) in various concentrations Pb on *Marsilea crenata* for n =5

Week	[0 ppm]Pb **PD ± S. e** [mV]	[3 ppm]Pb **PD ± S. e** [mV]	[10 ppm] Pb **PD ± S. e** [mV]	[20 ppm]Pb] **PD ± S. e** [mV]	50 ppm]Pb **PD ± S. e** [mV]
week-5	-116.0 ± 0.7	-106.0 ± 1.4	-94.0 ± 0.7	-88.0 ± 1.1	-88.0 ± 1.1
week-6	-118.0 ± 0.6	-85.2 ± 0.5	-83.0 ± 0.4	-74.0 ± 0.7	-68.0 ± 0.3
week-7	-124.0 ± 0.7	-88.0 ± 1.2	-68.0 ± 0.6	-59.0 ± 0.5	-58.0 ± 0.6
week-8	-126.0 ± 0.1	-97.0 ± 0.2	-81.0 ± 0.1	-68.0 ± 0.1	-51.0 ± 0.1

Based on ANOVA, there had been significant differences between PD control and PD *Marsilea crenata* under each treatments during the 5th week to the 8th week. This result had shown that additional lead concentration in media growth caused different effects on leaf surface electrical PD of *Marsilea crenata*. The higher concentration of lead the lower leaf surface electrical PD (Fig 3). The normal range PD were 116mV-126mV, while the lowest were 51mV-58mV on 50ppm. The results were in the range of -120mV- 160mV for pea in normal conditions and in between -40mV- 60mV for broad beans in stress condition for Mg[7]. Reduced electrical PD might have been signs of contamination in *Marsilea crenata* leaves.

High lead contamination resulted in damage to various physiological and biochemical processes[10]. Toxic effects of Pb decreased wheat growth, yield, photosynthetic pigments and ion contents[2]. Reduced electrical PD of *Marsilea crenata*had shown that lead contamination had an impact on plant growth; on the other hand, plants also had a mechanism to detoxify lead[17]. Reduced leave surface's electrical PD due to increased Pb concentration was shown to be the ability of *Marsilea crenata* in absorbing lead, which might have been accumulated in its root, shoot and leaves and might have caused disturbance in ion transportation activity.

Lead accumulation in plant root might have reduced the plant's ability in absorbing nutrients from the media and resulted in reduced ions in the plant. Reduced ion transportation affected reduced electrical PD[18, 19]. Those effects were caused by the removal of Pb from the media by the *Marsilea crenata*plant roots that might have increased Pb content on the plant leaves. Plant Pb content were increased with increasing concentration of Pb grown at 10 and 20 days old. Pb inhibits chlorophyll synthesis by causing impaired uptake of essential elements such as Mg and Fe by plants. Mg and Fewere important in the photosynthetic activity. The photosynthetic activity of plant was also influenced by many factors such as stomatal cell size, number of stomata, stomatal conductance and leaf area[20]. Lead might have impacted the photosynthetic process due to stomata and chlorophyll contents destruction in leaves. Reduced chlorophyll content would inhibit electrical energy to respond to light[21].

The negative effect of Pb on plant growth was accompanied by an increase in declining PD in plant leaves. Increment declining PDin plant leaves is a criterion for stress response, which is indicative by plant. Stress symptom on *Marsilea crenata* were observed through the following visual changes such as dark colouring of stalks,staining of leaves or stem; leaf browning; leaf red-brownish discolouration,and leaf chlorosis. Differences in the degree of expressed stress due to various Pb-concentrations applied to the root medium, the duration of treatment and to the Pb-concentrations had led to transition of leaf chlorosis into yellowing and necrosis of leave tips (Table 2). From our results, it was shown that detection of lead stress using biolectricity measurement was shown to have earlier results than using leaves measurement and visual observation. This led to the conclussion that biolectricity measurement could be used as an early detection on Pb stress on *Marsilea crenata*.

3.3. The Prospect of Marsilea crenata as a phytoremediation for an early detection of Pb Stress

The result had shown that stress on *Marsilea crenata* due to Pb contamination in growth media could be detected by measuring biolectricity response on leaves during photosynthetic process. Increased Pb in the growth mediaand exposure duration also has affectedroot growth. Accumulated Pb concentration on the root medium might have enhanced the degradation of chlorophyll[3]; reducing photosynthetic pigments and ion content[2]. High levels of Pbinhibited enzymes activities, alterations in membrane permeability, and disturbed mineral nutrition[3]. The longer exposure of Pb would cause more Pb ions entering the root of *Marcilea crenata*, and absorbed by the plant. The more Pb ions entering the plant's root meristem cells during exposure might induce DNA damage in root nuclei[22]. The effect on root growth depended on the concentration and duration of exposure and species.

The concentration of Pb in rice grain had significantly increased with increasing Pb in soils and the Pbavailability in soil. Root growth and shoot growth were reduced when rice seedlings were raised in sand culture for10 and 20 days in nutrient medium containing Pb[4]. Whereas Pb content in rice washigher in the roots than shoot[3,9]. High concentrations of Pb decrease germination in rice seeds and reduced the growth of seedlings[3]. Wild *Marsilea crenata*naturally grew in rice fields culture. Pb stress symptoms in *Marsilea crenata*were able to give an early warning to the farmer that the rice grown might contain toxic heavy metal Pb. In this case the *Marsilea crenata*should be used as phytoremediation to reduce therisk of Pb poisoning in soil and through the rice grown, entering the food chain. So the question remains, can *Marsilea crenata* possibly do the job to fill the gap in the phytoremediation process?.

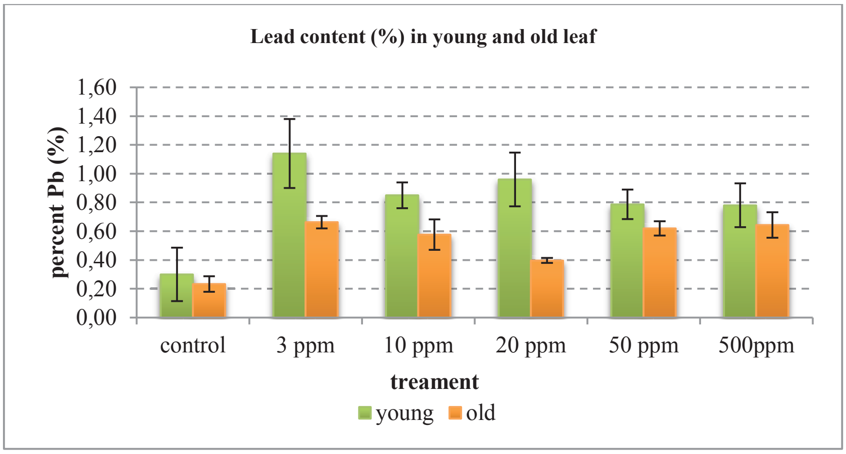


Fig.4. Lead content in young and old leaves of *Marsilea crenata* during one week exposure of Pb

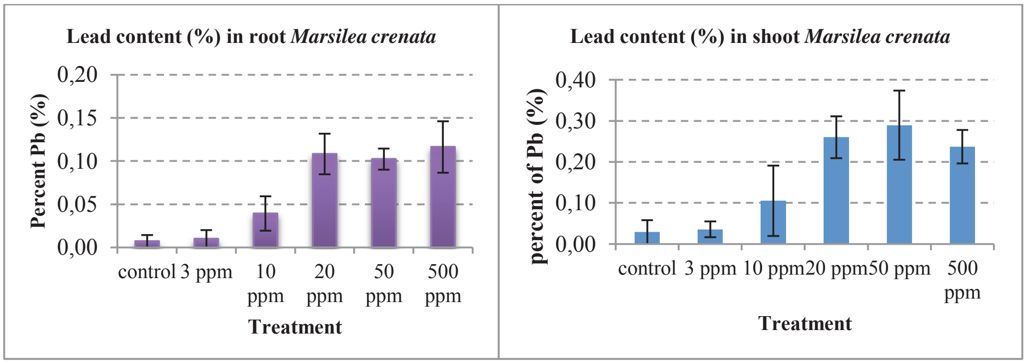


Fig.5. Lead content of *Marsilea crenata* during one week exposure of Pb a) in root, b in shoot

Using another sample from other locations in around Jember University and grown hydrophonically in two weeks, then after treatment in one week, the Pb contents were measured using AAS. The results had shown that the sample (control) containedan average of around 0.32% of their dryweight of Pb in their leaves in young leaves, and 0.23 % in old leaves during one week exposure. Interestingly the Pb content in 3 ppm were around 1.14% of their dryweight, which were highest among other treatment such as 10ppm, 20ppm, 50ppm and 500ppm. Declining Pb content in young leaves showed the concentration dependency, while in old leaves the content showed differently (Fig.4).

The capability of *Marsilea crenata* to absorb Pb were greater in its leaves than in its shoot and root. The comparison of Pb content in leaves is ≥ shoot ≥ root (Fig.4 and Fig 5). Except on 500 ppm, the increasing concentration in media seemed to increase Pb content in root and shoot of *Marsilea crenata*. The result had shown that young leaves accumulated more than old leaves. Leaves differed in their abilities to accumulate Pb depending on age[3], and leafy vegetables accumulated much higher contents of heavy metals[23] compared to other vegetables because leafy vegetables were most exposed to environmental pollution due to large leaf surface area. Previous reseachers reported that *Marsilea crenata*had the ability to absorb Pb around 0.404 mg/g at 12.5ppm concentrations, this exceeded the permissible limit according Indonesian regulation[24]and FAO/WHO-codex alimentarious commision, 2011[25]for lead total might not exceed the the 0.3 mg/kg for leavy vegetable.

Futher research is needed to measure on greater lengths such as in 3 consecutive weeks. Most planted rice seedling were 21 days old and during those times most rice field were prepared, so *Marsilea crenata* ccould be grown as phytoremediation.

Fig. 6.(a) Regrowth of *Marsilea crenata* after the death plant; (b) the root of *Marsilea crenata*.

The capability of *Marsilea crenata* to accumulate lead in roots and remove them to the leaves and shoot (Fig.4, Fig 5); a less damaged root of *Marsilea crenata*(Fig 6) apparently would support the regrowth of the plant. This would enable the plant to translocate some of the toxic lead ions out of the roots and concentrate them in the shoots and leaves which then died and were later replaced by regrowths from healthy portions of the root. Continuing process of death and regrowth would then take the excess lead to be removed from the soil for growth of healthy plants.

Low cost and easy maintenance maked the *Marsilea crenata* attractive to use as a viable treatment for municipal waste water phytoremediation, such as in *Lemna*[26], *Salvinia*[27]and Azolla[28]to clean up Pb contaminated environment and improving the environment quality. Together with biolectricity methods[7], it could be used to monitor the effect of lead stress and prosperously utilized in the line of phytoremediation[29] and biosorption[30] work and would also benefit in overcoming limited financial resources in developing countries and the lack of technical knowledge for farmer in remediation, and weak implementation of environmental policies.

Removing lead from the environment might reduce heavy metal toxic hazard. There was no safety threshold of lead poisoning to be found. It affected the haematological system even at low concentration, it might cause cognitive dysfunction, neoro-behavioural disorders, neurological damage, hypertension and renal impairment; causing in brain, heart, kidney, reproductive organdamage, increasing risk for childhood aplastic anaemia; induce effect on cell membranes, DNA and antioxidant defence systems of cells[1,31,32,33,34]. With the current cost of childhood lead poisoning to be US$43 billion per year. The cost benefit analysis for every US$ 1 spent to reduce lead hazards, there is a benefit of US$17-220. Lead poisoning accounts for about 0.6% of the global burden of disease[35]. Refering the detrimental effect and the cost, it seems that many farmers in Indonesia will continue facing the big risk, due to the huge cost where many people are still alive on US$1 pay per day. Using *Marsilea crenata* and biolectricity measurement for early detection of Pb stress as a part of phytoremediation system indication for Pb, contaminated semi aquatic environment will have a reduced remedial process cost and save the agricultural production especially in rice cultivation. *Marsilea crenata* posseses some properties of an ideal plant used in phytoremediation, such as being easily available in large amounts in nature, quick growth, and obtained free because it naturally grows in rice fields. Using *Marsilea crenata* as a part of phytoremediation also benefits in monitoring rice growth.

4. Conclusion and Recomendations

The result had shown that increased Pb concentration tended to inhibit plant growth. It was concluded that the method was viable as an early detection for the remediation process to protect agricultural economy and human security.Reduced lead from the environment reduced the risks caused by the highly toxic heavy metal. Continued monitoring of soil, plant, and water quality together with preventing metals from entering vegetables was a prerequisite in order to prevent potential health hazards to humans. Periodical monitoring of the rate of contamination and consumption were thus necessary to assess the overall exposure levels in the community. Reduced crop contamination and improved food safety could be achieved throughreducing pollution at it's source.

It is suggested that the usage of *Marsilea crenata* for daily intake should be avoided due to its tolerance to moderate amounts of Pb. Leaves may still be green but biolectricity has shown that the plant correlated with the accumulation of Pb for long periods. It also warns farmers that the rice may have been contaminated by Pb since *Marsilea crenata*alsogrows on the same field. Vegetables containing Pb if consumed for long periods may cause health related disorders. It is suggested to regularly monitor leafy plants and rice by observing any symptoms of Pb contamination. If *Marsilea crenata* has shown symptoms of chlorosis, consumption of the plant together with the rice should be avoided.

Further research is needed. The research has shown that *Marsilea crenata* may show a high accumulation of Pb if grown in a nutrient solution but may not show in a naturally Pb-contaminated soil. Better understandings of *Marsilea crenata* stress due to Pb contaminated soil may also reduce the risk of rice being contaminated by Pb since *Marsilea crenata* grows abundantly during the rice growing season. By knowing the existence of Pbsymptoms in *Marcilea crenata,* it will give an early warning to the farmer that the rice may have been contaminated by Pb. Caution and awareness will prevent larger damage on future rice production and in turn reduce the risk of people eating contaminated rice. Without remediation, high soil lead levels will never return to normal.

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