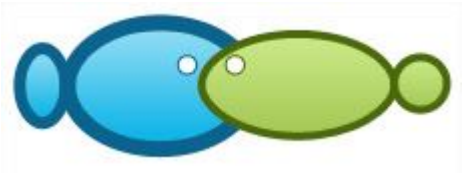

Accumulation and deposition of lead heavy metal in the tissues of roots, rhizomes and leaves of seagrass *Thalassia hemprichii* (Monocotyledoneae, Hydrocharitaceae)

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Abstract. *Thalassia hemprichii* was used to study the accumulation and deposition of lead in tissues of roots, rhizomes and leaves, as well as their impact on changes in the anatomy of that tissue. This plant was exposed to lead from $Pb(NO_3)_2$ solution at the concentrations of 0, 5, 15 and 25 ppm for 4 weeks. Lead was absorbed and deposited in apoplast in the cell walls and in the spaces between cells in tissues of roots, rhizomes and leaves. Anatomical structure analysis shows that the cell wall of endodermis and exodermis root layer became thick along with the increase of lead concentration. Air spaces in the cortex of roots and rhizomes widened. Cell wall thickening occurs also in the epidermis and endodermis of rhizome also at cuticle and epidermis of leaves. In general, changes in the anatomy of the roots, rhizomes and leaves followed to the increasing of lead concentration. This is one strategy for minimizing the translocation of lead in other tissue of *T. hemprichii*.
Key Words: anatomical responses, Pb, histochemical detection, marine plant.

Introduction. Lead is a heavy metal which is known to be highly toxic to humans (ATSDR 1993). Mutagenic and carcinogenic effects have been reported by Gerber et al (1980). The main source of lead in the environment is a component of the group of alkyl lead that are used as a gasoline additive, partly derived from waste disposal of industrial batteries, ammunition, protective material in wire, dyes, house paint and the burning of fuel for vehicles (Fardiaz 1992). Because of the toxicity of lead, then the behavior of lead in the environment and uptake by plants has been the subject of many researches. In plants, lead induces a decrease in chlorophyll synthesis, inhibiting the process of photosynthesis, decrease absorption of minerals, and change in the structure and permeability of the cell membrane (Seregin & Ivanov 2001; Singh et al 1997; Lamhamdi et al 2013; Hadi & Aziz 2015).

Thalassia hemprichii is a dominant seagrass in Indonesian waters. It is known that seagrass is an important ecosystem for the life of the various types of marine life including fish. Seagrass functions as a nutrient provider, as a spawning ground, a nursery ground and a feeding ground for a variety of marine life including fish (Aswandy & Azkab 2000). Based on previous research in waters of Ambon Island, lead metal was found in *T. hemprichii* ranging between 17.435-18.630 ppm on the roots, 4.849-7.558 ppm on the rhizomes and 7.225-8.439 ppm on the leaves (Tupan et al 2014). The presence of lead content in the parts of seagrass proves that there is a response of this plant towards the metal lead contaminants as well as an evidence of absorption and accumulation of this metal in the parts of seagrass. This also shows quantitatively that there is a content of lead on parts of *T. hemprichii*. However, there has been no qualitative research that indicates lead metal on the seagrass plant tissue. Various studies have been done on some of terrestrial plants and focuses on the roots as an organ of the plant that absorbs the lead within the soil (Tung & Temple 1996; Baranowska-Morek & Wierzbicka 2004).

Seagrass as marine angiosperms interact with sediments through the roots and rhizome as well as with the column of water through the leaves (Romero et al 2005). Therefore the accumulation and distribution of lead were not only found on the roots, but also found on the rhizomes and leaves. The aims of this study were to determine the uptake, translocation and deposition of lead in tissues of roots, rhizome and leaves of *T. hemprichii*, as well as their impact on change in the anatomy of the tissues.

Material and Method. *T. hemprichii* was maintained in an aquarium for 4 weeks, starting from 12 November 2012 to 10 December 2012. Samples of seagrass of the species *T. hemprichii* used in this study was seagrass with the number of leaves and vertical internodes look uniformly the same and in the healthy condition. These plants were exposed to lead from $Pb(NO_3)_2$ solution at the concentrations of 0, 5, 15 and 25 ppm for 4 weeks. Observations were made at week four. To complete the discussion, we used secondary data obtained from the research in journals and reports.

Anatomy observations of root, rhizome and leaves of T. hemprichii. Three plants were taken from each treatment and then separated according to the root, rhizome and leaves, which were then cleaned with distilled water and soaked in a solution of FAA (formaldehyde acetic acid alcohol, which serves as a fixative) for 24 hours. The aim of the fixation was to obtain the same tissue as the original one, and then to make semi-permanent preparation. Preparation was made according to the method of Ruzin (1999), which has been modified. Plant parts that have been fixed in FAA solution were than cut as thin as possible using microtome (Euremex MT.5503 clamp on hand microtome), then immersed in 1% safranine dye. After staining with safranine, the sections were mounted on the glass object. Analysis of the prepared slides was done by using a light microscope.

Parameters measured were profile anatomical structure of roots, rhizomes and leaves. Measurements were conducted on the thickness of exodermis and endodermis of roots, epidermis and endodermis of rhizome, abaxial and adaxial epidermis and cuticle of leaf. These observations were conducted using a Olympus CX 22 binocular microscope.

Observations on accumulation and deposition of lead in tissues of roots, rhizomes and leaves. The observations of lead on plant tissue were made using histochemical methods which are considered to be fast, simple and accurate (Glater & Hernandez 1972). Sodium rhodizonate is a specific good chromophoric reagent which gives red color with a buffer solution at pH of 2.8. Sample preparation was carried out according to modified method proposed by Glater & Hernandes (1972); Tung & Temple (1996) and Baranowska-Morek & Wierzbicka (2004). Seagrass parts (roots, rhizome and leaves) of each treatment were fixed by the FAA for 24 hours, then cut thin with a microtome (clam on hand microtome). Thin sections are then soaked in a solution of sodium rhodizonate 0.2% for 30 minutes, then one drop of acetic acid buffer solution of pH 2.8 was added. Tissue samples were then rinsed with distilled water and placed in an object glass and observed on a microscope.

Statistical analysis. Data were analyzed with ANOVA of multifactorial analysis ($p < 0.05$), and to determine the significance differences between treatments with the least significant difference (LSD) test. Analysis of data was performed using GenStat version 14.

Results and Discussion

Lead accumulation on root tissue. Root anatomical structure of *T. hemprichii* showed some changes in the tissue affected by expose to the lead. Exodermis and endodermis tissue were thickened at the treatment of high lead concentration exposure (Table 1).

Table 1

Changes in the anatomical structure of roots (µm) at different concentrations of lead (ppm)

	Concentration of lead (ppm)			
Root tissue	*0*	*5*	*15*	*25*
Exodermis thickness	2.138±0.348[a]	2.375±0.339[ab]	2.470±0.343[b]	2.700±0.405[c]
Endodermis thickness	1.000±0.177[a]	1.063±0.242[a]	1.150±0.271[b]	1.250±0.250[b]

Note: Value are mean±SE (n = 3) and differences between means were compared by Fisher's least significance test. Different letters indicate significant differences at $p < 0.05$.

The higher the concentration of lead causes the thicker the exodermis and endodermis roots cells (Figure 1). It was indicated that the accumulation and contamination of lead can accelerate the maturation of exodermis and endodermis cell wall. The loss of root epidermal layer and its replacement by exodermis occur in some plants such as *Ranunculus* sp., where exodermis acts as a coating (Raven et al 2001). In the middle of cortical cells, it would be seen that the radial plate separating air spaces was absorbed. Consequently, the air spaces seem bigger. Changes in the shape and organization of cells show a disruption of heavy metals in the impair maturation in the roots caused by heavy metals ability to disrupt the balance of hormones (Barcelo & Poschenrieder 1990; Sandalio et al 2001).

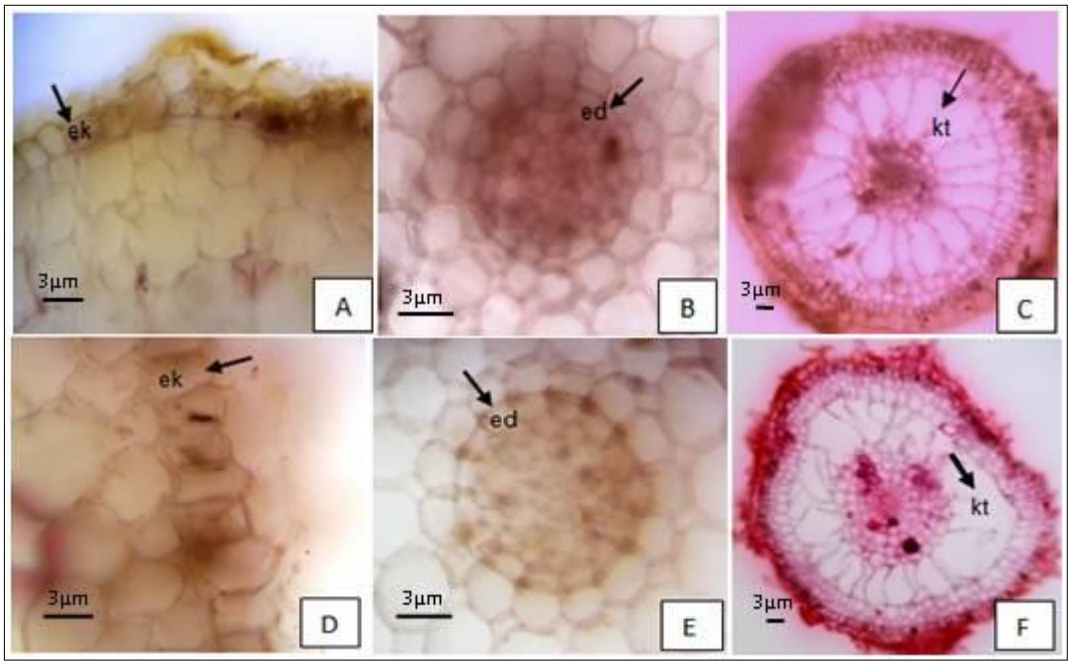

Figure 1. Changes in the anatomical structure of roots: A, B, C = control; D, E, F = 25 ppm Pb treatment (ek: exodermis, ed: endodermis, kt: middle cortex).

Exodermis and endodermis thickening in *T. hemprichii* was also an indication of strategy of this plant in minimizing the lead translocation. In apoplast, exodermis and endodermis play an important role in protection against various kinds of stress experienced by the plant (Enstone et al 2003). The same was also reported by Lux et al (2004) which stated that the exodermis and endodermis root tissue had a high proportion in characterizing high tolerance to heavy metals. Exodermis and endodermis of *Brachiaria decumbens* have a good ability to restrict the flow of metal in the apoplast, and thickening of cell wall in the root to provide areas for retention of heavy metals, thereby reducing the metal translocated to the leaves (Gomes et al 2011).

Lead accumulation at rhizome tissue. Analysis of the anatomical structure of the rhizome showed some changes in epidermis and endodermis tissue and thickened cell based on treatment concentration (Table 2) and (Figure 2). Thickening layer of the

epidermis as a result of the absorption of lead which participated in with nutrient allegedly resulted in earlier maturation of cells. In addition to the deposition of lead on the cell walls, also lead to thickening of the tissue. Thickening of the cells of the endodermis was also an adaptation to prevent the translocation of lead into the stele.

Table 2

Changes in the anatomical structure of rhizome (µm) at different concentrations of lead (ppm)

	Concentration of lead (ppm)			
Rhizome tissue	*0*	*5*	*15*	*25*
Exodermis thickness	2.678±0.495[a]	3.036±0.620[b]	3.406±0.481[b]	3.643±0.627[b]
Endodermis thickness	1.538±0.169[a]	1.678±0.372[a]	1.750±0.316[a]	2.133±0.376[b]

Note: Value are mean±SE (n = 3) and differences between means were compared by Fisher's least significance test. Different letters indicate significant differences at $p < 0.05$.

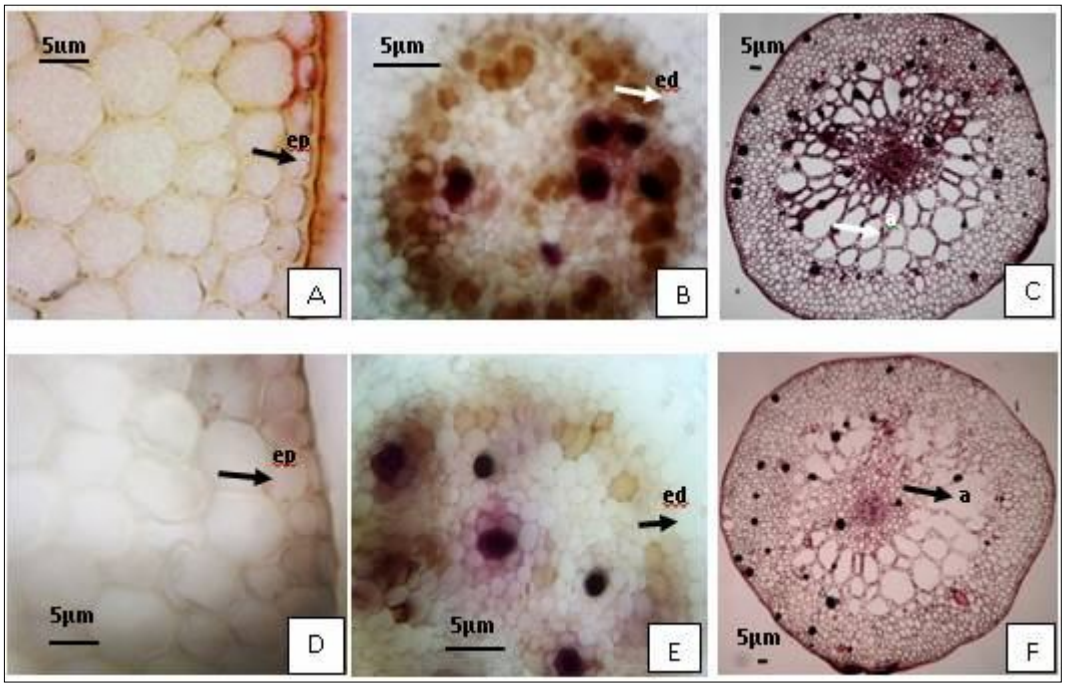

Figure 2. Changes in the anatomical structure of rhizome: A, B, C = control, D, E, F = 25 ppm Pb treatment (ep: epidermis, ed:endodermis, a: air space).

Cortex cells in rhizome had seen to change in air space. Air space was enlarged due to reduceding of diaphragm. These changes may be due to interference of lead bound to the cell walls of the cortex diaphragm and lead disruption to the hormonal balance. Changes in size, shape and arrangement of cortical parenchyma cells was due to the ability of heavy metals in disturbing the balance of hormones (Gomes et al 2011).

Al-Saadi et al (2013) found changes in the cortical parenchyma and air space stems of aquatic plants *Potamogeton* sp. and concluded that the change was due to the metal binding to the cell wall and the space between cells, and shows the relationship between metal tolerance in wetland plants against the loss of oxygen to the anatomy of the stem. Furthermore, they reported that the formation of the air space was one of the important strategies of wetland plants acclimatization to the conditions under water, which not only facilitates the transport of oxygen in the plant but also increases the potential loss of oxygen to the rhizosphere.

Lead accumulation at leaves tissue. Leaves anatomical structure analysis shows a change at cuticle layers and thickening at epidermis cell according to treatment concentration. Cuticle layers and leaves epidermis became thicker as lead accumulation increases (Table 3) and (Figure 3).

Table 3

Changes in the anatomical structure of leaves (µm) at different concentrations of lead (ppm)

Leaves tissue	*Concentration of lead (ppm)*			
	0	*5*	*15*	*25*
Cuticle abaxial thickness	0.325±0.087[a]	0.375±0.094[a]	0.438±0.149[a]	0.650±0.114[b]
Cuticle adaxial thickness	0.381±0.262[a]	0.375±0.094[a]	0.438±0.149[a]	0.650±0.132[b]
Epidermis abaxial thickness	1.563±0.148[a]	1.838±0.122[ab]	2.000±0.094[b]	2.088±0.154[b]
Epidermis adaxial thickness	1.563±0.149[a]	1.838±0.129[a]	2.000±0.094[b]	2.075±0.154[b]

Note: Value are mean±SE (n = 3) and differences between means were compared by Fisher's least significance test. Different letters indicate significant differences at $p < 0.05$.

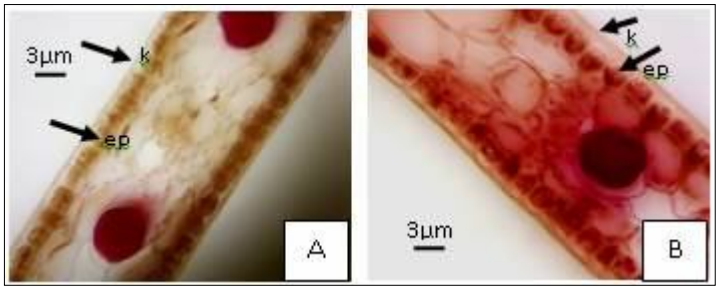

Figure 3. Changes in the anatomical structure of leaves: A = control, B = 25 ppm Pb treatment (ep: epidermis, k: cuticle).

Water plants submerged in the water can absorb water and soluble materials including metals through the body, including the leaves, and this absorption is influenced by the structure and permeability of the cuticle. The entry mechanism of lead particles into the leaf tissue is through the stomata of the leaves since stomata leaves are larger than lead particle size. This is turn cause lead easily fit into the leaf tissue through a process of passive absorption. *T. hemprichii* is a seawater plant that does not have stomata but has a thin cuticle and hollow oval as the inclusion of the carbon material for the process of photosynthesis (Tomlinson 1980; Kuo 1983). The cuticle acts as the entry point where lead goes through the leaves into the body of seagrass, that indicated cuticle thickening occurs because lead is trapped in the cavity of the cuticle.

Deposition of lead at root tissue. Staining sodium rhodizonate gave pink-purple, red-brown to blackish brown in tissues that accumulate lead (Tung & Temple 1996; Baranowska-Morek & Wierzbicka 2004). The appearance of red stains found in two forms as diffusive or as granular deposit. At the root tissue could be seen lead on the root hairs, epidermal cells, exodermal cells, cortical layer, endodermal cells and at the vascular conducting systems (Figure 4). The granular form of lead found in the cell walls, and at the spaces between cells. Lead color intensity that appears depended on the concentration of lead accumulated.

There were differences in color intensity between the control and treatment of lead concentration, where the higher concentrations of lead accumulated, the color intensity became stronger. Lead was first entered into the root hairs absorbed by the roots and into the epidermal cells. At the hair roots strong visible red color was seen in diffusive form, then in the epidermal cells. The deposits of lead were predominant on the cell walls and the spaces between cells. Deposition of lead in apoplastic seems to be the main mode of deposition in root tissue (Tung & Temple 1996). Lead will continuously be absorbed and transported into the cell wall, and the amount of lead deposition increases with the increase of lead concentration.

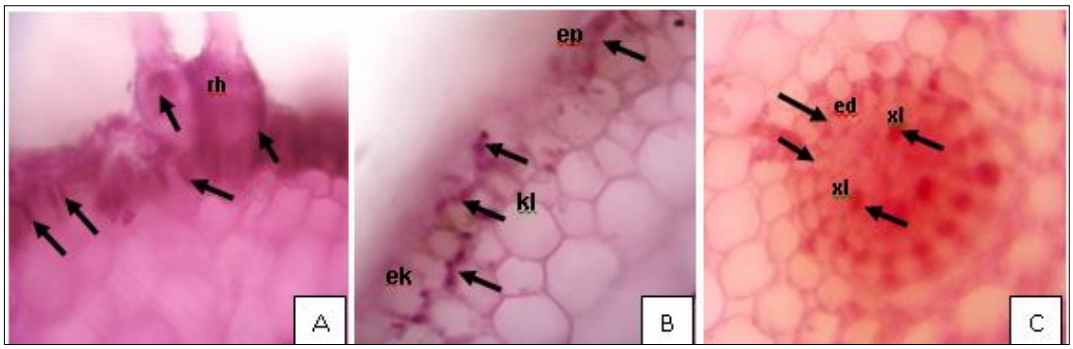

Figure 4. A, B, C = deposition of lead (arrow) on the root tissue with sodium rhodizonate (rh: root hair, ep: epidermis, ek: exodermis, kl: outer cortex, ed: endodermis, xl: xylem).

According to Zimdahl (1976) and Koeppe (1977), it seems that the deposition of lead tied to the middle lamellar polysaccharides. They also showed that the diffusion of lead deposits was restricted. It was only diffusive smaller size of lead particle was transported to the cell wall fluid, whereas the larger sized were deposited directly to the cell wall. The amount of lead in the roots of displacement however, depends on the physiological status of the plant. On healthy plants, mineral translocation in roots was greatest when the rate of growth, osmotic pressure and transpiration rate is high (Barber 1995). Lead absorption will take place in line with the absorption of nutrients by the roots passively and directly absorbed by the cells of the epidermis including the hair root. The most active absorption was performed by the primary cell wall of epidermal cells in the meristem region, especially at the mitotic phase of the root meristem (Tung & Temple 1996).

The deposit of lead was seen in endodermal cells especially on the casparin strip with dark red color (Figure 4). The endodermis with casparin strip becomes a good barrier against the flow of substances through it to the stele. Due to effect of casparin strip, the lead translocation from endodermis to stele will be prevented. Casparin strip barrier effect is clearly resulting in the prevention of translocation of lead to the stele and further into the vascular tissue, however, the movement of lead through endodermal cells on thin-walled parts can still take place. According to Tung & Temple (1996), translocation of lead occurs through water transpiration, lead absorbed from the root zone and can not pass through the lignified casparin strip on endodermis, but entering through the cells that are not lignified on endodermis. Therefore, the movement of lead in the cortex toward the center stele may take place. Lead is also transported through the xylem and phloem that constitute water transportation lane and the main result of photosynthesis in plants.

Deposition of lead at rhizome tissue. Lead on the rhizomes showed red deposit which spread on a cuticle and epidermal layer (Figure 5). Lead that goes along with water and nutrients through the cuticle and epidermis will accumulate on the cell walls, then passes through water transportation to the central stele. Afterward red stain was later seen in the cell walls of the diaphragm that separates the air spaces in the cortex. Deposit of lead seen on the cell walls, indicates the binding of lead on the cell walls causing the cells to become thicken.

As a results of the binding of lead in the diaphragm among the air spaces, these cells were easily to separate. So the air spaces become bigger. According to Al-Saadi et al (2013) the binding of metal on the walls of parenchymal cells and air space of cortical layer stem of *Potamogeton* sp. is a probable strategy of the accumulator species. Red stains were also seen on the central stele and vascular bundle. Metal deposition on the vascular bundle caused reducing of vascular bundle and enlarging the air spaces of protoxilem as well as enlarging the phloem sieve elements (Al-Saadi et al 2013).

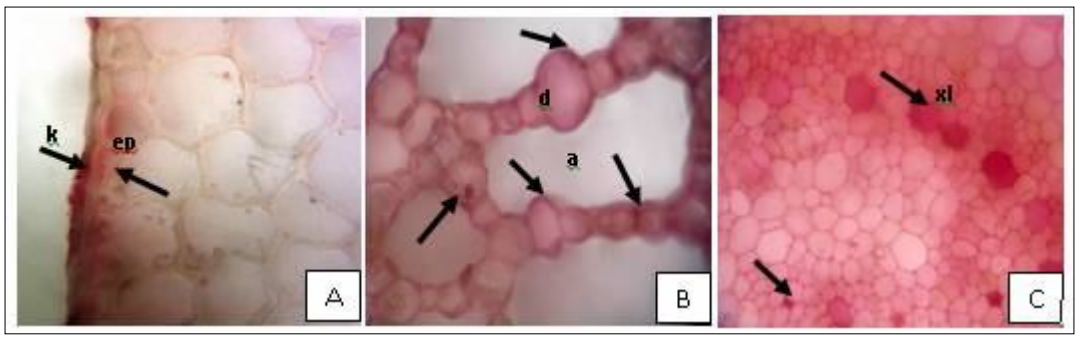

Figure 5. A, B, C = deposition of lead (arrow) on the rhizome tissue with sodium rhodizonate (k: cuticle, ep: epidermis, d: diaphragm, a: air space, xl: xylem).

Deposition of lead at the Leaf tissue. On leaves, red stain could be visible on the cuticle, the walls of the epidermal cells, mesophyll parenchyma, air spaces and vascular bundle (Figure 6). Lead that entered the leaf tissue through the cuticle was marked with red deposit in the layer, then went to the epidermal layer and adsorbed on the cell wall of the epidermis. The binding at the cell wall of epidermis was used as a strategy to prevent that lead going further into the chloroplast cells. If the lead entered the chloroplast cells, it can disrupt the system of CO_2 fixation in the process of photosynthesis (Larkum et al 2006). Red deposit was next seen on the walls of mesophyll parenchyma cells. Exposure of heavy metals on plants will lead to a reduction in the size of mesophyll cells, and these disorders can lead to the collapse of the parenchymal tissue of the palisade and the spongy tissue (Sridhar et al 2005).

Red deposit of lead was also seen at the air space and leaf vascular bundle. It was indicated that lead was distributed in the air chamber and then to the vascular bundle which then translocated to the stems and roots through phloem in the vascular tissue. Al-Saadi et al (2013) reported that the reduction in thickness of the leaves was due to the reduction of epidermal cells and the aerenchyma. Furthermore, it was also reported that metal deposition is also observed in the cells which would reduce the vascular bundle. Reduction in the number of elements of the xylem in response to heavy metal was an adaptation to maintain the flow of water on the plant (Sandalio et al 2001).

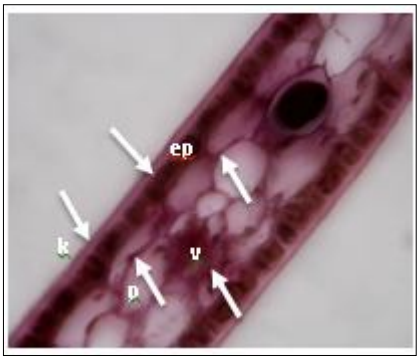

Figure 6. Deposition of lead (arrow) at the leaves tissue with sodium rhodizonate (k: cuticle, ep: epidermis, p: parenchyma, v: vascular bundle).

The impact of lead accumulation in seagrass to fish and marine environment. Seagrass beds have a very high primary and secondary productivity and supports the abundance and diversity of fish that utilize seagrass at some stages throughout their life (Gilanders 2006). *Siganus* spp. are a group of fishes that often found living in the area of seagrass bed. This was related to feeding habits of *Siganus* spp. which are herbivores, eating seagrass and epiphytic that associated with seagrass (Latuconsina et al 2012; Ambo-Rappe 2010; Munira et al 2010; Karnan et al 2016). Some seagrass species which are eaten by the fishes are *Enhalus acoroides*, *T. hemprichii*, *Halophila ovalis*, *Cymodocea rotundata*, *Halodule uninervis*, and *Syringodium isoetifolium* (Merta 1982).

Absorption of heavy metals in marine waters can occur indirectly in the food chain, where seagrass as a primary producer is the beginning of the food chain, and it will be eaten by animals like fishes, so that the heavy metals in the seagrass will move to the fishes. Research conducted by Zainury et al (2011) found that lead accumulates in the liver of *Siganus* spp. through the food chain, where these fish eat the seagrass that has been contaminated of lead. According to Sahetapy (2011), the lead, which entered the body of juvenile fish, would be xenobiotic. This could inhibit the action of acetylcholinesterase (AChE) resulting in accumulation of acetylcholine (ACh) in the central nervous system. The accumulation will induce tremors, incoordination, and causes death.

On the other hand, due to the ability to absorb heavy metals, seagrass can reduce heavy metal pollution in marine waters. This plant can be used as the first level indicator to monitor the heavy metal elements in coastal environments (Prange & Dennison 2000). Short & Wyllie-Echeverria (1996) in Oliva et al (2012) stated that seagrass is very sensitive to changes in the environment and in particular to the impact of human activity. Sensitivity to environmental change is based on the fact that seagrass interact directly with a column of water through the leaves and sediment through the roots and the rhizomes (Romero et al 2005). This is shown by the response to the anatomy of seagrass *T. hemprichii* showing good adaptation to survive in a polluted environment of lead heavy metals, so that this plant can be used as good bio-indicator on waters. Some researches on seagrass as bio-indicators have been done on the species *Zostera marina* (Krause-Jensen et al 2005), *Posidonia oceanica* (Romero et al 2005; Gobert et al 2009; Royo et al 2010), *Cymodocea nodosa* (Oliva et al 2012), *Cymodocea rotundata* (Herawati 2008).

Conclusions. *T. hemprichii* had the ability to absorb and accumulate lead on the tissue of roots, rhizomes and the leaves hence become good bioaccumulator as well as bio-indicator for lead heavy metal pollution due to its specific anatomical responses to the pollutant.

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