Ultrasonic Extraction for Estimating Bioavailability of Phosphorus in Particulate Forms

（懸濁物質中の生物利用可能性リンの超音波抽出法による定量）

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Doctor of Engineering

NGUYEN MINH NGOC

グェンミンギョク

豊橋技術科学大学

Toyohashi University of Technology

Abstract

Phosphorus (P) is an essential nutrient for plant growth and required to maintain profitable crop and livestock production in the prevalent intensive agricultural plans. However, its excessive use has caused undesirable growth of algae and cyanobacteria in aquatic environment, leading to the harmful eutrophication which is blamed for mortality of aquatic culture in closed water areas and threats to human health, particularly during the summer period. In order to control human-caused eutrophication, acceptable levels of total phosphorus (TP) were limited in the water quality standards in many countries but it includes all of P species. Some of them are complex forms that do not either feed algae or contribute to eutrophication. Hence, the term of bioavailable P (BAP) appeared to define the P fraction which is readily available for algal uptake and really contribute to eutrophication. Regardless of the difference in chemical forms, in environment P can be separated by centrifugation or filtration into two states: dissolved phosphorus (DP) and particulate phosphorus (PP). DP is generally considered readily available for algal uptake, whereas PP is partially bioavailable. P has a strong affinity to particulate matter, thus PP bound to sediment and soil particles comprises the majority of P in surface runoffs flowing either into drains or overland into aquatic environments. Therefore, an understanding of the BAP fraction in PP bound to soils and suspended sediments, especially related to agricultural sources, is necessary for better management of eutrophication in a watershed. Recent reports have also alleged the involvement of bioavailable phosphorus (BAP) in particulate forms in the severity of eutrophication. However, current methods that can estimate particulate BAP are too time-consuming and meticulous. They require longer than 14 days for incubation in bioassays, 4 days for the sequential extraction scheme or 17 hours for single step-extraction using mechanical shaking, respectively. Thus, the amounts of analyzed samples are very limited. It leads to the demand of a new method which require less time for determining accurate BAP concentration in PP. A possible solution is using ultrasonic treatment to accelerate the transformation of P into extracts. Recently, ultrasonic treatment has been studied as an efficient extraction technique that takes less working times as well as improves yields and quality of the extracts from food and environmental samples. In this study, we investigated an extraction using 0.1 M NaOH solution in combination with an ultrasonic treatment to quantify the potential BAP in particulate forms, especially in soil and suspended sediment from river water related to

agricultural sources.

The first study used Sharpley's extraction with the mechanical shaking in 17h as the conventional method to examine the BAP concentrations in our target samples of soil and suspended sediment. Next, we evaluated our proposed ultrasonic method by comparing with Sharpley's method. The most optimal working conditions of the ultrasonic treatment were defined at which, the BAP extracted by ultrasonic treatment was similar to those obtained by the conventional extraction. The investigated working conditions of ultrasonic treatment included intensity, extraction time and ratio of sample to extractant. Finally, we evaluated the bioavailability of P obtained from the extractions by bioassays for the growth of P-starved *Microcystis aeruginosa*.

We quantified BAP concentrations in soil and suspended sediment samples collected from a representative agricultural field in Umeda River basin. Umeda River was selected because it flows into Mikawa Bay which has been reported one of the most eutrophic regions in Japan's main island. Our study was compatible with the previous study about the proportion of BAP in PP in agricultural streams. It confirmed the potential risk of P pollution in Umeda River basin and Mikawa Bay come from agricultural sources.

The most optimal conditions for ultrasonic treatment were identified. The proposed method allowed for an extraction time of only 1 min whereas the mechanical shaking method requires 17 hours for BAP extraction. The extraction process is less time-consuming than alternative conventional methods and permits analyses of a greater number of samples.

The growth of algae in the media containing samples after the ultrasonic extraction was at an identically similar level with those in the media containing samples after conventional extraction. It reinforced the notion that ultrasonic treatment could provide a similar quantification for BAP in soil and suspended sediment samples when using conventional extraction. The high correlation between the amount of extracted BAP and algal growth at the stationary phase of incubation suggested that the BAP fraction could be entirely obtained in a single extraction. Additionally, The limiting effect of P on the algal growth was confirmed. Although bioassays observed the growth of algae in cultures using samples after extraction as the sole phosphorus (P) source, we confirmed that the remained P was able to be in algal cells. We suggested that when nutrients, especially P, were deficient, the algae could utilize cellular nutrients for their growth. Thus, using *M.aeruginosa* would properly be inappropriate for accurate AGP tests to evaluate the bioavailability in P depleted environments.

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Nguyen Minh Ngoc

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Chapter 1 Introduction

1.1. Background: Eutrophication and sources of phosphorus inputs to aquatic environment

Water is an important resource for human but clean water is scarcely available. Serious problems with water pollution further reduce clean water supply greatly. One of the challenging problems over the world nowadays is the wide spreading eutrophication in aquatic environment not only in freshwater ecosystems but also in coastal areas. The common phenomena of eutrophication are blue-green algal blooms and red tides depending on the phytoplankton species living in that watershed. With severe eutrophication, hypoxic conditions occur, then disrupt ordinary ecosystem and furthermore create "dead zone" where no life can be sustained (Grattan et al., 2016; Diaz and Rosenberg, 2008). Eutrophication itself is a process that occurs in a water body as a response to increased levels of nutrients. In nature, eutrophication is a common phenomenon and a part of the gradual aging process of many freshwater ecosystems over many centuries (Chislock et al., 2013). However, this process has been artificially boosted by human activities, and then the water ecosystems eventually suffer the excessive increase of nutrients beyond what the natural ability of ecosystem can handle.

Nitrogen (N) and phosphorus (P) are the major nutrients that cause eutrophication and other harmful impacts associated with nutrient enrichment (US National Research Council, 2000). Both N and P control should be considered in an eutrophication management strategy but the role of P has attracted more attention. Because the ratio of P in plant content to its availability in water environment is larger than the ratio of N, P is usually more important than N in limiting the growth of algae and phytoplankton.

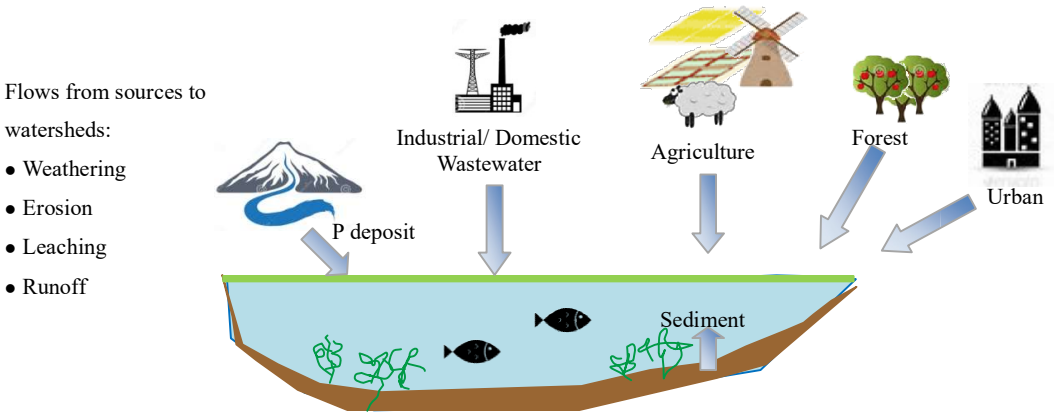

Figure 1-1. Major sources of phosphorus pollution

Under typical freshwater conditions where physical factors are contributive to the growth of algae, additions of P to the system are more likely to lead to accelerated growth than additions of N. Especially, due to the characteristics of short supply in rivers and other freshwater ecosystems, P is generally the limiting nutrient in these environments (Fox et al., 2016, Zienlinski and Jekatierynczuk-Rudczyk, 2015, US National Research Council, 2000). Moreover, the blue-green algae can obtain the N they require from the atmosphere through the chemical process of nitrogen fixation, thus theoretically creating a nearly limitless N source for these aquatic ecosystems.

Now, the sources of phosphorus causing eutrophication of water bodies are being discussed. The threshold of P concentration in receiving water causing eutrophication is 0.03 mg L^{-1} dissolved P or 0.1 mg L^{-1} total P (Brady and Weil, 1996). All activities in the entire drainage area of watershed have potential affects to water quality in several direct and indirect ways (*Fig.1-1*). P may be released from phosphate deposits during weathering, erosion and leaching as well as from bottom sediments during seasonal overturns. Otherwise, excessive P may come from anthropogenic pollution sources.

Point sources include discharges from industrial and domestic wastewater treatment plants as well as agricultural point sources such as confined livestock units. Non-point or diffuse sources include excess runoff from forest, urban areas and agriculture fields. In recent years, many techniques have been implemented to control point-source pollution, thus it became easier to identify or reduce contamination from point sources than from diffuse sources. Therefore, more researches have been directed towards non-point (or diffuse) sources (Dupas et al., 2015, Monteagudo et al., 2012), primarily from agricultural activities. Intensive agricultural fields often use phosphate-containing fertilizers as an excellent soil amendment to improve soil quality for crop production. This practice results in P accumulation in soils and can increase the risk of P transport to nearby watershed through leaching, erosion and runoff process. P accumulation in watershed promotes a proliferation of aquatic plants, especially simple algae and cyanobacteria. Over time, the water surface becomes muddy and typically green, yellow or red in color. The enhanced growth of these cyanobacteria and algal blooms disrupts normal function of the environment, despoiling oxygen required for respiration by fish and other species in water habitat. The over growth of algae may also inhibit algae and plants in under layer from sunlight which is essential for photosynthesis. When these algae and plants die and decompose, oxygen depletion happens and progresses to hypoxia. The hypoxic conditions lead to suffocation death of species under the water such as shrimp, fish and other aquatic biota. In extreme cases, the anaerobic conditions encourage the growth of bacteria that produces toxins that may cause human illness and mortality of birds, mammals following consumption in food web; thereby it can bring about aquatic dead zones and lessens biodiversity in that area (Grattan et al., 2016; Diaz and Rosenberg, 2008).

Table 1-1. Acceptable levels of phosphorus in some countries

Country	Freshwater (mg / L)	Coastal water (mg / L)
Japan [1]	•Conservation Lakes & Reservoirs (class V) [1a]: 0.10 (TP) • Water supply (class I) [1a]: 0.005 (TP)	•Fishery and recreational water (class V) [1b]: 0.09 (TP)
USA [2]	•All fresh water (American Samoa): 0.15 (TP) •Warm fresh water (Basin 3) (California): 0.20 (TP)	•Oceanic water (American Samoa): 0.01 (TP) •Coastal water (Virgin islands): 0.05 (TP)
CANADA [3]	•Streams: 0.03 (TP) • Lakes: 0.02 (TP)	
AUSTRALIA [4]	•Aquatic ecosystem protection: 0.10 (TP)	•Salinity environment:0.05 (TP)
PHILIPPINES [5]	•Public water supply (class A): 0.1 (PO_4-P)	•Recreational water (class B): 0.2 (PO_4-P)

TP: Total phosphorus; PO_4-P: phosphate

[1] Environmental quality standards for human health – Ministry of the Environment, Government of Japan, 2001; [a] Conservation of the water environment, Chapter 3; [b] Environmental standards of water quality, Chapter 7;

[2] Office of Water Regulations and Standards – Environmental Protection Agency, United States, 1988;

[3] Canadian Water Quality Guidelines for the Protection of Aquatic Life – Canadian Council of Ministers of the Environment, 2004;

[4] Australian and New Zealand guidelines for fresh and marine water quality – Department of the Environment, Australian Government, 2000;

[5] DENR Administrative Order No.34, Department of Environment and Natural Resources, Philippines, 1990

1.2. Forms of phosphorus in environment and pollution from agricultural sources

In the efforts to prevent human-caused eutrophication, the limits on the concentration of total phosphorus (TP) allowed in aquatic environment (summarized in *Table 1-1*) have been placed in the environmental quality standards in many countries (Rattan et al., 2016, van Puijenbroek et al., 2014, Okada and Peterson, 2002). Although TP has been described in environmental quality standards, it is not really an accurate eutrophication indicator. It covers all of P species (*Table1-2*) including complex forms (such as carbonate and apatite bound P, some of organic humic P, mineral organic P, etc.) which are not available for the growth of algae and cyanobacteria. Hence, the term of bioavailable phosphorus (BAP) appeared to define the P fraction which is readily available for algal uptake and really contribute to eutrophication.

Regardless of the difference in chemical forms, in environment TP can be separated by centrifugation or filtration into two states: dissolved phosphorus (DP) and particulate phosphorus (PP). The former DP includes inorganic phosphorus generally in apatitic minerals or secondary precipitates formed with Ca, Fe and Al and free phosphate ions

Table 1-2. P species in water environment (Pacini and Gachter, 1999).

Fractionation scheme	Soluble Reactive P	Non-Reactive P
Step1	Immediately available inorganic P	Other immediate available P
Step2	Redox-sensitive P bound to Fe, Mn	Organic P
Step3	P bound to Al, Inorganic P compounds soluble	Microorganism-P, Polyphosphates, Detrius organic P, Humic P
Step4	Carbonate and Apatite bound P	Organic P
Remains	Organic and Mineral, non-extractable, refractory P	

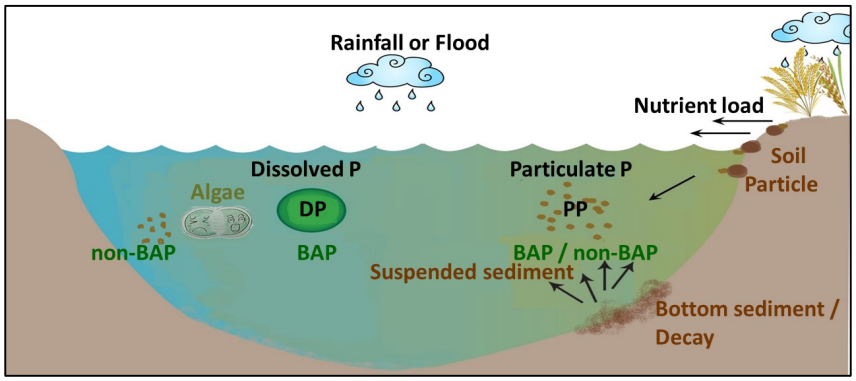

Figure 1-2. P forms in water environment and the importance of BAP.

(PO_4-P: $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}) attached to sorption surfaces or dissolved in soil water, organic phosphorus such as sugar phosphates, phosphoproteins, mononucleotides and inositol phosphate, nucleic acids, phospholipids, teichoic acid and aromatic compounds and small fraction less than 0.45 μm. PP includes precipitates of P, P adsorbed to manure particles and amorphosus P.

DP is generally considered readily available for algal uptake, whereas PP is partially bioavailable (*Fig.1-2*). P has a strong affinity to particulate matter, thus PP bound to sediment and soil particles comprises the majority of P in surface runoffs flowing either into drains or overland into aquatic environments. Especially, when rainfall or flood occur, rain flows transport P associated with soil particles to watershed and P bound to sediment become suspended, leading to the predominance of PP in river water. Therefore, an understanding of the BAP fraction in PP bound to soils and suspended sediments, especially related to agricultural sources, is necessary for better management of eutrophication in a watershed so as to determine correctly the potential of eutrophication and water pollution and react accordingly. Recent reports have also alleged the involvement of bioavailable phosphorus (BAP) in particulate forms in the

severity of eutrophication (McDowell et al., 2016, Ellison and Brett, 2006, Sharpley et al., 1991).

1.3. Problem statement

1.3.1 Current methods for quantifying particulate BAP

In many years, several methods have been suggested for determining bioavailability of particulate phosphorus in soil (*Fig.1-3*). Among them, algal growth potential (AGP) test is believed to be the most reliable. AGP test has been accepted as a standard method for determining the effects of phosphorus enrichment to water bodies because it can investigate the direct influence of phosphorus on the test algae (Ronald and Donald, 1987, Miller et al., 1978, Thomas Shoaf, 1978). This method is based on the principle that maximum yield is proportional to the amount of nutrients which are present and bioavailable in minimal quantity in respect to the growth requirements of the test alga. However, it is too time-consuming. It requires 7 to 100 days for incubation only (Donald et al., 1994, Miller et al., 1978), and thus the number of conducted test is limited in a given time. This method is therefore not really suitable for routine lab analysis.

In order to shorten the working process, chemical extraction methods was developed with an aim to categorize soil P into different species that respond similarly to changes in natural environment. Single extraction methods are used to give an estimation of a certain form of P while sequential extraction methods aim to characterize P in more detail and quantify P into forms that are separated by some chemical property (*Table 1-3*)

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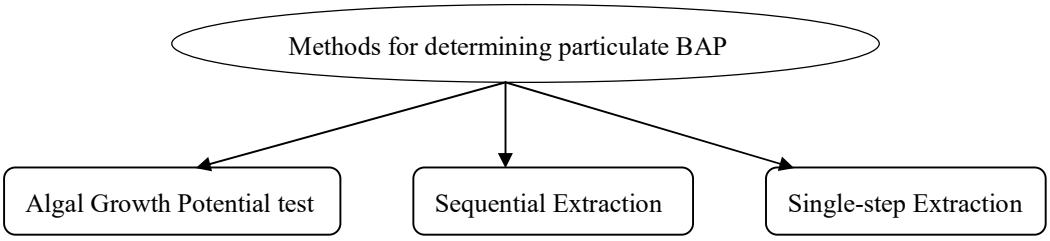

Figure 1-3. A summary of methods for determining BAP in particulate form

Extraction procedures are often studied simultaneously with biological tests to examine the correlation between extracted P fraction and P uptake by algae. Olsen et al. (1954) first introduced bicarbonate extraction ($NaHCO_3$) for predicting bioavailable P in calcareous soils. Since $NaHCO_3$ is used to decrease the Ca^{2+} activity by forming $CaCO_3$, phosphorus is more easily extracted as soluble in supernatant. However, in acid soils, the solution pH which was buffered to pH 8.5 promotes desorption of P. Also, in soils containing Al^- and Fe^- bound phosphorus, the P concentration in solution increases as the pH increases because the higher concentration of OH^- anions decreases the ability of PO_4-P to compete for sorption sites (Soinne, 2009). This method is widely used in European countries such as Denmark, England and Australia, New Zealand while the Mehlich-1 test is widely used in the United States of America. Mehlich (1953) suggested "double-acid"extractant using 0.05 M HCl and 0.0125 M H_2SO_4 for determining the amounts of phosphorus in acid, low cation exchange capacity soils. Sagher et al. (1975) demonstrated that amounts of phosphorus in surface soils assimilated by *S.capricornutum* in 28-day incubations originated in the fraction

extractable with 0.1 M NaOH (Dorich et al., 1985). Sharley et al. (1991) afterward showed that 0.1 M NaOH extractable phosphorus was closely related to the growth of several algal species when using the ratio of soil to solution (1:500) (w.t./w.t.) (Pierzynski and Sharpley, 2000, Sharley et al. 1991). In most recent studies, the phosphorus concentrations extracted by single-step extraction using 0.1 M NaOH are believed strongly correlated with algal-available P fraction in general soil (Pierzynski and Sharpley, 2000, Andre et al., 1996, Sharley et al. 1991).

Concurrently, the development of sequential extraction procedures has expanded in order to quantify phosphorus in more detail. The basis of this method is to sequentially extract several phosphorus compounds from the same soil sample by using extractants with increasing strength (Turner and Cade-Menun, 2005). The fractions that are extracted first have higher bioavailability while the fractions that are extracted last have the lowest bioavailability. A variety of sequential extraction schemes has been suggested on the basis of reactivity of a particular P phase in each given extractant. The most common schemes for determining phosphorus in soils are outlined in *Table 1-3*. Bowman and Cole (1978) proposed a sequential extraction scheme based on chemical solubility. In this scheme, the labile extracted by 0.5 M $NaHCO_3$ and the moderately labile fraction extracted by 1.0 M H_2SO_4 were considered to be more readily bioavailable than the moderately resistant and highly resistant humic and fulvic extract with 0.5 M NaOH. Hedley et al. (1982) suggested a comprehensive scheme to quantify soil phosphorus also based on chemical solubility, and bioavailability was supposed on the basis of chemical stability. The labile P extracted in $NaHCO_3$ was assumed to be more bioavailable than those extracted by strong NaOH or H_2SO_4. In addition to

Table 1-3. The common extraction schemes for determining phosphorus in soil

Scheme	Extractants	Designation
Olsen et al.	0.5 M $NaHCO_3$	Labile P, available P for plant growth Suggestion for soil pH > 7.4; Need to buffer to pH 8.5 if soil pH < 5.0.
Mehlich	0.05 M HCl + 0.0125 M H_2SO_4	Labile P, available P for plant growth Simultaneous multi-element extraction of K, Ca, Mg in soil.
Sharpley et al.	0.1 M NaOH	Labile P, immediately available P P proved equal to BAP determined by algal growth potential test.
Bowman and Cole	i.0.5 M $NaHCO_3$	Labile P
	ii. 1.0 M H_2SO_4	Moderately labile P
	iii. 0.5 M NaOH	Moderately resistant and highly resistant P
Hedley et al.	i.Anion exchange resin	Labile P
	ii. 0.5 M $NaHCO_3$	Labile P
	iii. Fumigation, 0.5 M $NaHCO_3$	Microbial P
	iv. 0.1 M NaOH	Iron- and aluminium-bound P
	v. 0.1 M NaOH + sonication	Inter-aggregate P
	vi. 0.1 M HCl	Calcium-bound P
	vii. Digestion, concentrated H_2SO_4 and H_2O_2	Residual P
Pacini and Gachter	i.1 M NH_4Cl	Labile P, immediately available inorganic P, loosely adsorbed P
	ii. 0.11 BD	Redox-sensitive P bound to Fe and MN, organic P
	iii. 1 M NaOH	Humic substance bound P and poly-P
	iv. 0.5 M HCl	Carbonate and apatite bound-P, acid labile organic P

the procedures developed for soil fractionation, Pacini and Gachter (1999) developed another sequential scheme which can be used for trace sources whereas the amounts of non-apatite particulate phosphorus extracted in 1.M NH_4Cl, 0.11 M BD and 1 M NaOH, respectively, may be considered potentially bioavailable.

1.3.2 Potential of ultrasonic treatment for phosphorus extraction in particulate matters

Recently, ultrasonic treatment has been studied because it represents an efficient technique for extracting various elements or chemicals from food and environmental samples that takes less time than previous methods, thereby improving sampling yields and the quality of the extracts (Gurkan et al., 2016, Wu and Zhu, 2016, Salemi et al., 2013, Rondano and Pasquali, 2008, Dolatowski et al. 2007). Ultrasonic waves have frequencies above human-hearing sound (20 Hz to 20 kHz) but below microwave frequencies (300 MHz to 300 GHz). This technique is based on the penetration of ultrasonic waves into materials (Dolatowski et al. 2007), combined with their ability to transfer energy to attached substances such as P, thereby weakening the bonds with particulate matter and increasing release of the substances into the extractant. Despite its potentiality, its application to quantitative studies of BAP has been limited. This creates an idea that we can test ability of ultrasonic sound to rapidly extract P from soils and suspended sediments.

1.4. Objectives of this study

In efforts to control harmful cultural eutrophication, bioavailable phosphorus (BAP) in should be more concerned than total phosphorus (TP) due to its real impact on

cyanobacterial and algal growth. Previous studies have reported BAP in particulate is a great potential source for P pollution, and it becomes dominant in river water when rainfall or flood occurs. Several methods have been suggested for estimating particulate BAP but they are too time-consuming thus not yet suitable for frequent monitoring. Although ultrasonic treatment is potential, its applicability on BAP quantification is still uncertain. In this view, the overall objective of this research has been to develop a simple method that can quantify BAP in particulate forms not only quickly but also accurately. In order to archive a method, the following targets were considered:

(1) To examine the BAP fraction in particulate forms from agricultural sources including soils and river suspended sediments.
(2) To consider the suitability of ultrasonic treatment for estimating particulate BAP.
(3) To verify the bioavailability of P obtained from ultrasonic treatment to confirm if that P is really BAP.

1.5. Structure of the thesis

This thesis is organized into six chapters:

Chapter I

The thesis starts with the general introduction about phosphorus and important roles of BAP in eutrophication management; background reviews of conventional methods for quantifying particulate BAP; and explains the objectives of this study to test a simpler method for BAP analysis.

Chapter II

In this chapter sampling sites, sample collection, methodology and analyses are described in detail.

Chapter III

The investigation of ultrasonic treatment's applicability on extracting particulate BAP has been explained. The most optimal working conditions of the ultrasonic extraction are proposed.

Chapter IV

The preliminary evaluations of phosphorus obtained from the ultrasonic extraction to algal growth potential tests are described in this chapter.

Chapter V

The bioavailability of phosphorus after the extractions is verified, and the relationship between the BAP obtained from extractions with the algal growth are illustrated.

Chapter VI

The overall conclusions and recommendations of the three-year doctoral research project are compiled in this last chapter.

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Chapter 2 Methodology

2.1. Outlines

To archive the overall objectives, the following issues were considered:

- BAP in soil and suspended sediment samples was estimated using the conventional Sharpley's extraction (1991).
- The optimal working conditions of proposed ultrasonic treatment for BAP extraction were investigated. The BAP values obtained from ultrasonic treatment were compared with those extracted by conventional Sharpley's method in order to identify the ultrasonic treatment's most optimal working condition.
- Algal growth potential (AGP) test was conducted using the soil and suspended sediment samples as the sole P source to confirm the "real" bioavailability of extracted P.

This chapter explains more detail about each of sampling and analytical methods which supported the aforementioned target methodology.

2.2. Sampling site

Mikawa Bay is one of the most eutrophic regions in Japan's main island. We selected Umeda River (about 22 km in length, with a basin area of approximately 86 km^2) which flows into Mikawa Bay through Toyohashi City, Aichi Prefecture as the study site (*Fig.2-1*). The watershed includes forest (1.6% of land use) in the upstream, intensive agriculture (97.5%), especially cabbage fields at which Toyohashi is one of the leading producers in Japan, and urban area (0.9%) in the mid- and downstream. In the agricultural activities surrounding this watershed, phosphate fertilizers are intensively used for growing crops. That has exacerbated nutrient pollution of adjacent bodies of water and has led to the widespread eutrophication in Mikawa Bay (Rasul et al., 2014).

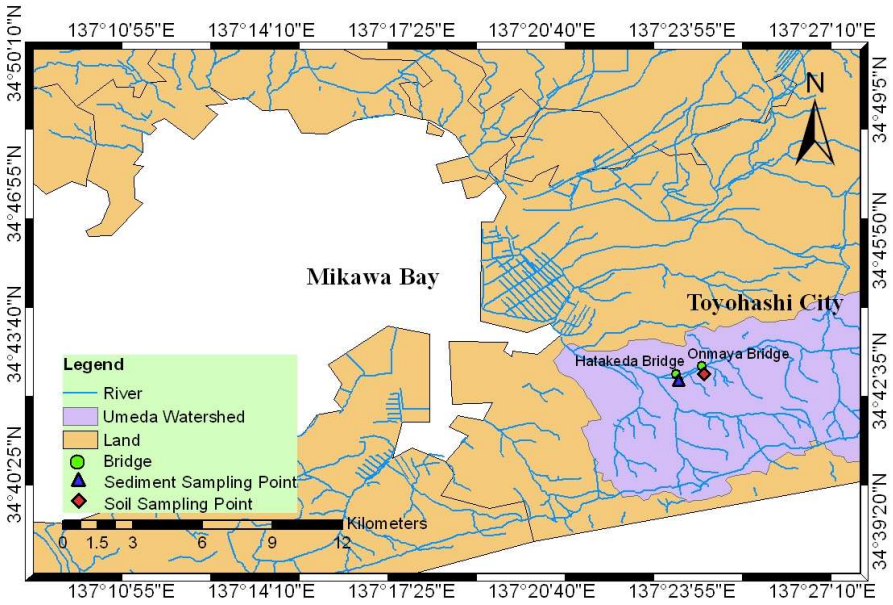

Figure 2-1. Umeda watershed and locations of the sampling sites.

A watershed is more easily eutrophied when it situates in or nearby a fertile area, thereby we collected representative samples of soil from a Chinese cabbage field in river basin. Soils were taken on 2011 January 26th in a depth of 0 to 50 mm from a fertilized Chinese cabbage field located approximately 200 m southwest of Onmaya Bridge (approximately 6.3 km upstream from the river mouth) (*Fig.2-1*). The depth of collected soil was chosen to represent an average effective depth of surface soil–runoff interaction for a range of soils, rainfall intensities, slopes, and soil management characteristics. The locations in the cabbage field were chosen randomly. The collected soils were combined to produce a single homogeneous sample to be representative of the surface soil in the study area.

Suspended sediments were concentrated from river waters. We collected river water samples manually immediately after storm events on 2014 September 5th, 11th and

October 6th when the suspended sediment was well mixed. River water was obtained by dipping a bucket into the river from Hataketa Bridge, near the water quality monitoring system (4.9 km upstream from the river mouth) (*Fig.2-1*). The collected sediments were representative of suspended load in the study river system in the autumn.

2.3. Sample pretreatment and preservation

Immediately after collection, all samples were transported to the laboratory. In order to minimize P degradation during storage, the suspended sediment samples were concentrated by continuous-flow centrifugation at average speed 196,000 m s^{-2} for 250 mL/min (Hitachi Koki, Tokyo, Japan; Himac CR22G high-speed refrigerated centrifuge; R18C continuous rotor) and decantation. The centrifuge intensity was applied to separate suspended sediment from river water that was not sufficient to separate colloidal particles from the supernatant. Then the combined soil and concentrated sediment samples were air-dried at 40°C for three days. All samples were sieved through a 0.149-mm mesh screen to remove small particles such as plant fibers and colloids of decayed humic matters, and then stored under refrigeration.

2.4. Extraction methods

2.4.1 Conventional mechanical shaking extraction

Sharpley's extraction (*Fig.2-2a*) was chosen as the conventional method to quantify the BAP concentrations in the target samples. This method was first suggested by Dorich et al. (1985) and proved to be similar to BAP determined by algal growth potential tests (Sharpley et al., 1991). BAP concentrations in soil and sediment samples were determined by a single-step extraction (Dorich et al., 1985) in 0.1 M NaOH using

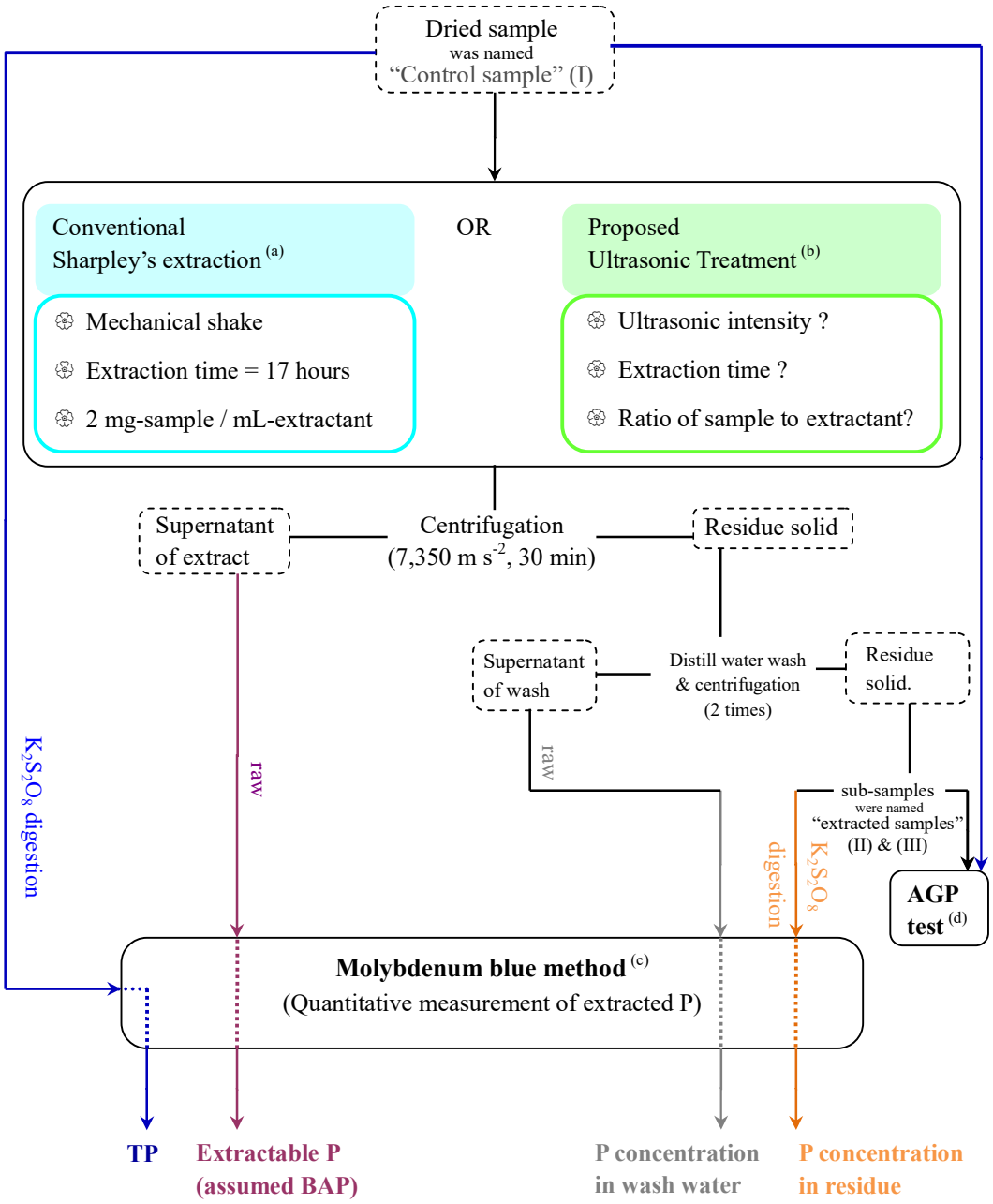

Figure 2-2. Procedure for particulate BAP measurement including a comparison between the two extraction methods.

conventional mechanical shaking for 17 hours. This method was demonstrated effectively estimate BAP in agricultural runoff in Oklahoma, USA at a ratio of soil (or suspended sediment) to extractant of 2 mg mL^{-1} using 0.1 M NaOH as extractant (Sharpley et al., 1991). 100 mg of each sample (soil or sediment) was placed in a 50-mL centrifuge tube with 50 mL of 0.1 M NaOH, shaken mechanically for 17 hours on a multi shaker (EYELA, Tokyo, Japan; MMS-210 orbid multi shaker) at ambient temperature.

2.4.2 Proposed ultrasonic extraction

To shorten the extraction time and improve the extraction efficiency, we tested a new method based on the use of an ultrasonic horn (Branson, Connecticut, USA; Ultrsonic Disruptor Sonifier II, model W-450) (*Fig.2-3a*). The horn has a fixed operating frequency of 20 kHz and can generate intensities ranging from 0 to 400 W. During treatment, the horn was placed 1 cm from the bottom of the container without contacting the walls of the centrifuge vial that contained the soil or river sediment sample in the extractant (distilled water or 0.1 M NaOH) (*Fig2-3c*), and which was fully immersed in an ice-water bath to prevent a temperature increase (*Fig2-3b*). Firstly, the extraction conditions were varied to investigate the optimal working conditions for the following parameters: the ultrasonic intensity, ratio of soil or sediment to solution volume, and extraction time (*Fig.2-2b*). Each treatments were performed for three independent samples (i.e., n = 3). Next, we fixed the working conditions at the identified optimal values, and again conducted the ultrasonic extraction in parallel with the mechanical shaking method in order to evaluate alternation of the two methods by statistical tests.

(a) (b) (c)

***Figure 2-3*.** Equipments were used in ultrasonic extraction. (a) Sonifier front view; (b) proposed assembly in our experiment; (c) the horn was placed 1 cm from the bottom of the experimental vial.

2.4.3 Phosphorus analysis

The concentrations of P transferred to the solution phase obtained from the extractions were measured colorimetrically on neutralized extracts by molybdenum blue method (*Fig.2-2c*). This method (Murphy and Riley, 1962) has been used as a standard method for determining the concentration of extracted P. It is based on the reaction of PO_4-P with molybdate to form a blue compound. The intensity of the color corresponds to the P concentration in solution and can be measured by a spectrophotometer (Bran and Luebe TRAACS 800 Autoanalyzer, Norderstedt, Germany). Extracts are the clear supernatant in each sample vial that were collected right after the extractions by centrifugation at 7,350 m s^{-2} (Kubota, Tokyo, Japan; model 5100 table-top centrifuge with an RS-4 universal swing rotor) for 30 min (*Fig.2-2*). The solid residue was washed twice with 50 mL of distilled water for 10 min each on the orbital shaker, and then separated from the wash water by means of centrifugation (7,350 m s^{-2}, 30 min)

(*Fig.2-2*). The clear supernatants of wash waters were pooled and analyzed to prevent underestimation of the extracted P as a result of secondary adsorption of the liberated P by solid surfaces in the residue (Ruttenberg, 1992; Pacini and Gachter, 1999), whereas the residues were preserved for the following AGP tests (*Fig.2-2*). To prepare samples for the P analysis, the extracts and wash waters were neutralized with 5 M HCl (NaOH extracts) or with either 1 M HCl or 1 M NaOH (all samples) and diluted (NaOH extracts, to 50% of the original concentration), and kept at 4°C in the dark until analysis.

The analysis of raw extracts measured the amount of soluble reactive extractable phosphorus in each sample while the analysis of digested samples measured the amount of total phosphorus (TP) extracted in each sample (*Fig.2-2*). The digestion with 20 mL of distilled water and 4 mL of potassium persulfate ($K_2S_2O_8$) (4%) in an autoclave (Sanyo, Osaka, Japan; MLS-3750) at 120 ºC for 30 minutes converted all of P forms into soluble P which can be detected by autoanalyzer. The quantity of non-extractable phosphorus remained in each samples was derived as the analysis of digested residue solid after extraction and twice washes (*Fig.2-2*). All measurements were the average of duplicates. The recovery rates were calculated by dividing the sum of the P in the supernatants and the P in the solid residues by the TP in the samples before extraction.

2.5. Algal growth potential (AGP) tests

2.5.1 Incubation procedure

The particulate residues after extraction (*Fig.2-2*) at the most optimal working conditions identified in *Chapter 3* were washed twice with distilled water to remove any remaining NaOH, then air-dried at 40 ºC, and preserved in refrigerator for bioassays to verify the bioavailability for algal growth potential. The soil and sediment samples

without any extraction were defined as “control sample” (I); the residue solid of samples extracted with ultrasonic treatment were termed extracted samples “with ultrasonic treatment” (II); and the samples extracted by conventional mechanical shaking for 17 hours were called “with mechanical shaking” (III) (*Fig.2-2*).

Prior to the evaluation of bioavailability, we prepared P-starved *Microcystis aeruginosa*. We collected algal cells from stock cultures (clone NIES 44, National Institute for Environmental Studies Collection, Japan) by centrifugation (7350 m s^{-2}) for 30 min. Then, we rinsed these cells twice with P-free CB medium (Kasai et al., 2004, Shirai et al., 1986) using the aforementioned centrifugation. We incubated the rinsed algal cells into P-free CB medium in 500-mL Erlenmeyer flasks for two weeks until the algae began using their intercellular P. The deficiency of P in the algae was indicated by a color fade of the culture from dark green to yellowish green.

We cultured the P-starved *M.aeruginosa* (1.3×10^5 cells mL^{-1}) into P-free CB media containing samples (respectively I, II and III) as the sole P source. *M.aeruginosa* was selected because it is one of the most common cyanobacteria responsible for blue-green algal blooms in fresh water and in low-salinity estuaries around the world (Okubo et al., 2012, Ren et al., 2016, Marinho et al., 2007). We also cultured the algae to P-free CB media containing no external P source for “negative controls”, whereas CB-media containing β-glycerophosphate were used for the “positive controls”. Incubation occurred in a growth chamber under cool white fluorescent light with a 12:12-h light: dark cycle at 25 ºC for 42 days. The microscopic cell count was conducted using a hemocytometer to observe the contamination of indigenous bacteria during incubation period. Growth yield was monitored by chlorophyll-*a* (Chl-*a*), suspended solid (SS), particulate organic carbon (POC), particulate organic nitrogen (PON), DP and PP using

samples taken on specific days during incubation. All measurements were the average of three independent replicates per sample.

2.5.2 Analyses of algal growth monitoring parameters

In order to analyze Chl-*a*, SS, POC and PON, DP and PP, 10 mL of each well-stirred sample medium was respectively passed through filter papers (GF/F glass microfiber filters φ 47 mm, Whatman, GE Healthcare, Tokyo, Japan) applying vacuum until the sample was dry. For the measurement of SS, POC and PON, DP and PP filter papers were prepared a day before by washing with distilled water, then dried at at 400 ºC for 2 hours to eliminate microfibers which can lead to the overestimation of the retained residue amount.

Chl-*a* was determined spectrophotometrically (US Environmental Protection Agency, 1991) (V-530 UV/VIS Spectrophotometer, JASCO Corporation, Japan) after extraction the filter papers with 10 mL of methanol overnight at -20ºC in the dark.

DP was measured by the autoanalyzer (Bran and Luebe TRAACS 800 Autoanalyzer, Norderstedt, Germany) as the soluble P in the raw filtrate, whereas PP was analyzed using the dry filter papers after digestion with 20 mL of distilled water and 4 mL of $K_2S_2O_8$ (4%) at 120 ºC for 30 minutes.

SS was determined as the difference in the weight filter papers before and after the filtration. The filter papers were dried at 105ºC for 2 hours (Gray et al., 2000).

POC and PON were determined by a NC analyzer (Sumigraph NC-22A, Sumika Chemical Analysis Service, Ltd. Tokyo, Japan) using the filter papers after measuring SS.

2.6. Statistics

A two-way analysis of variance (ANOVA) test was conducted to compare the effect of extraction methods (ultrasonic treatment versus conventional extraction, using distilled water versus 0.1 M NaOH) on the concentration of P extracted from four samples (soil versus sediments) (Ichihara, 1989). F-ratio in ANOVA test was used to evaluate the equality of mean values.

$$\mathrm{F} = \frac{\text{variation between sample means}}{\text{variation within samples}}$$

Probability p value was used to evaluate probability that there was no difference between extraction methods or sample types, or the interaction of extraction methods and sample types. It can take on a value from zero to one to express the chance that our hypothesis will occur. Zero means there is no chance that our hypothesis will occur whereas one means that the hypothesis is certain to occur. Numbers between zero and one quantify the uncertainty of the hypothesis.

A Pearson correlation coefficient r and linear regression (Ichihara, 1989) was measured to analyze the similarity between P extraction with the ultrasonic treatment and the conventional extraction methods at certain conditions (i.e. same ultrasonic intensity, ratio of soil or sediment to extractant, or extraction time) and the relationship between extracted P with the algal growth potential. The formula is:

$$r = \frac{\sum_{i=1}^{n}(x[i] - \bar{x})(y[i] - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x[i] - \bar{x})^2 \times \sum_{i=1}^{n}(y[i] - \bar{y})^2}}$$

where $x[i]$ and $y[i]$ are the raw samples from the two datasets that are to be correlated against, and $\bar{x}$ and $\bar{y}$ are their respective means.

Student t distribution, significance level α and probability p (Ichihara, 1989) were used to test the differences between the ultrasonic treatment and the conventional

mechanically extraction. A Student-*t* is calculated as:

$$t = \frac{\bar{x} - \mu}{(s/\sqrt{n})}$$

where $\bar{x}$ is the sample mean, μ is the population mean, s is the standard deviation of the sample, n is the sample size.

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Chapter 3 Optimal Working Conditions of the Proposed Ultrasonic Extraction

3.1. Outlines

In this chapter, the applicability of ultrasonic treatment for extracting particulate P was investigated. The P obtained from extraction was assumed to be BAP. Several parallel experiments were carried out to clarify the optimal conditions for the ultrasonic treatment at which the P extracted by ultrasonic treatment were the most similar to those obtained by the conventional mechanical shaking method. The similarity was evaluated by statistical analyses. The effects of ultrasonic intensity, ratio of sample (soil or suspended sediment) to solution volume, extraction time, and extractant type (distilled water versus NaOH) on the concentration of extracted P were investigated.

3.2. Investigation procedure

At first, the values of ratio of soil or sediment to extractant and extraction time were held at 2 mg mL^{-1} (Sharpley et al., 1991) and 5 min (Turner, 2008), whereas ultrasonic intensity was varied respectively 10, 15, 20, 30, and 40 W to select the optimal value. Next, the values of ultrasonic intensity and extraction time were fixed at the aforementioned optimal value and 5 min, whereas the ratio of soil or sediment to extractant was varied respectively 0.02, 0.10, 0.20, 0.50, 0.70, 1.0, and 2.0 mg mL^{-1} (Fuhrman et al., 2005). Finally, the values of ultrasonic intensity and ratio of soil or sediment to extractant was set at the optimal values, whereas the extraction time was varied respectively 1, 2, 5, and 15 min (Beizhen et al., 2008). Additionally, in order to extract the BAP forms that exist naturally in water the ultrasonic parameters with

distilled water (pH 7) as the extractant was also tested. The water-extractable P (WEP) has been shown to be the form of P that is immediately available for uptake by aquatic biota (McDowell and Sharpley, 2001). Due to extraction being slower with distilled water (Zhou et al., 2001), I a 30-min duration for the distilled water extraction was also included.

3.3. Samples

The proposed extraction was tested on four samples, including one soil and three suspended sediments: (a) Soil sample collected on 2011 January 26th; (b) Suspended sediment sample collected on 2014 September 5th; (c) Suspended sediment sample collected on 2014 September 11th; (d) Suspended sediment collected on 2014 October 6th. Extractions were conducted triplicates. P concentrations were the average of duplicate analyses.

3.4. Results and discussion

3.4.1 Ultrasonic extraction's working conditions

Firstly, in order to optimize the ultrasonic intensity, the ratio of the solid phase (soil or sediment) to the extractant (distilled water or 0.1 M NaOH) was fixed at 2.0 mg mL^{-1}, whereas the intensity was varied from 10 to 40 W. The results showed that with 5 min of extraction, increasing the ultrasonic intensity slightly increased the amount of extracted phosphorus (*Fig.3-1*). In both extraction treatments (using distilled water or 0.1 M NaOH), increasing the intensity above 30 W had no significant effect on the amount of extracted P. The intensity was not tested higher than 40 W to avoid loud noise (which can injure workers' hearing) and to minimize the risk of erosion of the ultrasonic horn.

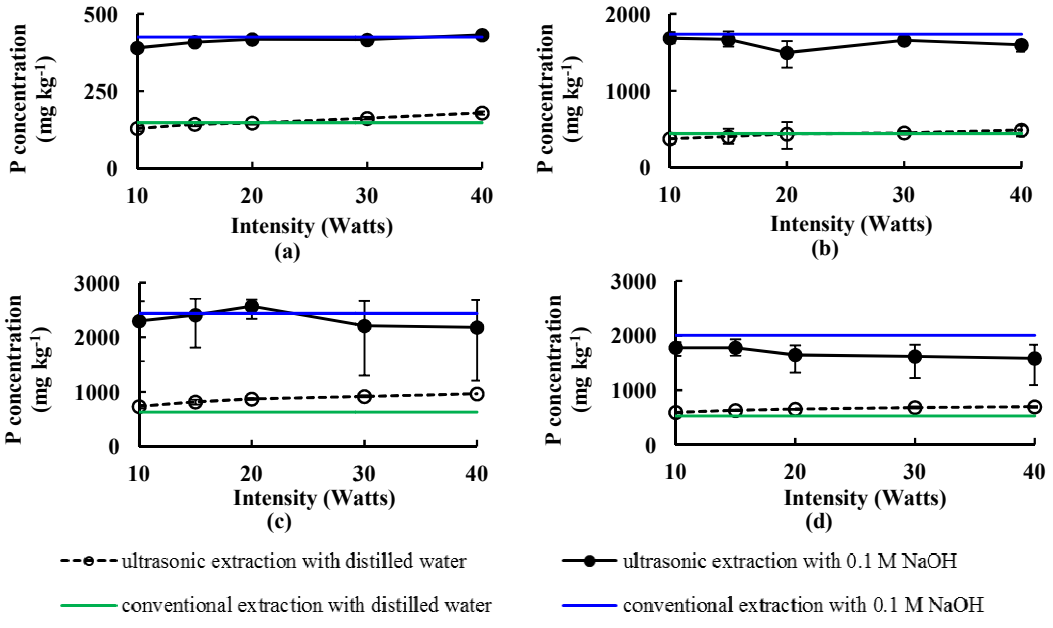

Figure 3-1. Effect of ultrasonic intensity on the amount of extracted P. Each value represented the mean of triplicates. Bars represent positive and negative errors.

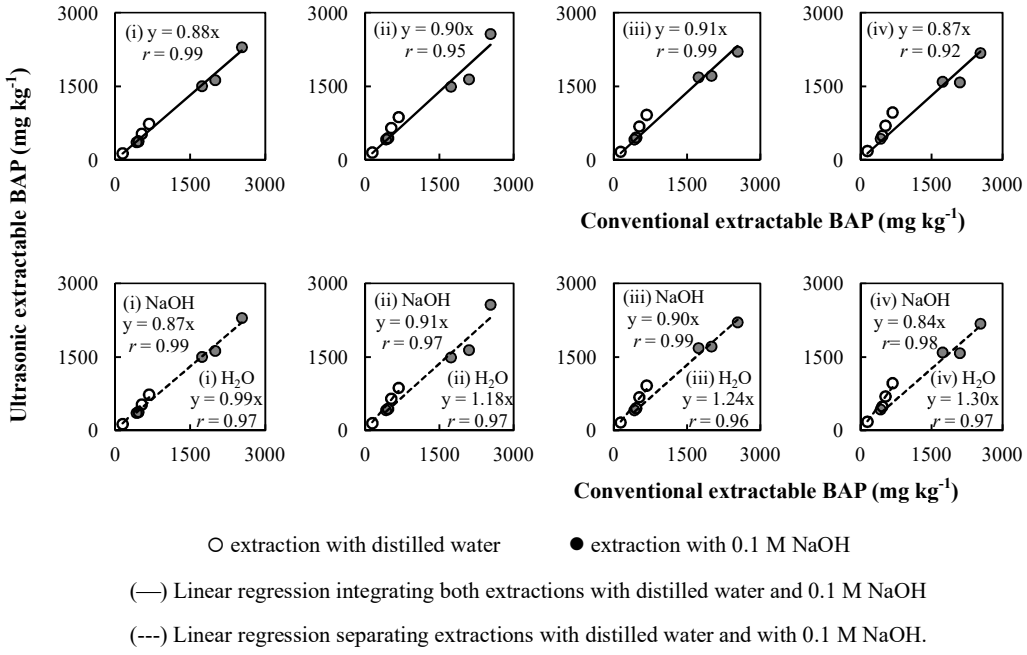

Figure 3-2. Pearson correlations between extraction methods when the ultrasonic intensity was 10 W (i), 20 W (ii), 30 W (iii), 40 W (iv) respectively.

In the water extraction of all soil and sediment samples (*Fig.3-1*), the extracted P rose marginally but similar to the P concentration obtained by the conventional mechanical shaking extraction (slope = 0.99 to 1.30, r = 0.96 to 0.97) (*Fig.3-2i to iv)*. In the NaOH extraction of sediment (b) (*Fig.3-1b*) and sediment (c) (*Fig.3-1c*), the P concentration fluctuated between 15 and 30 W of ultrasonic intensity. In the NaOH extraction of sediment (d) (*Fig.3-1d*), the P concentration gently fell when increasing the ultrasonic intensity from 15 to 20 W. However, in the most cases, the concentration of P obtained by the analysis at 30 W was the most similar (slope = 0.91, r = 0.99) (*Fig.3-2iii*) to the values extracted by the conventional mechanical shaking method. It was compatible with the statistics using data of NaOH extraction individually (slope = 0.91, r = 0.97) (*Fig.3-2iii*). Thus, 30 W was chosen as the optimal ultrasonic intensity.

Next, the effect of seven samples (soil or sediment) to extractant ratios with the optimal ultrasonic intensity of 30 W and 5 min of extraction time was examined (*Fig.3-3*). The P concentration was generally the highest at the smallest ratio (0.02 mg mL^{-1}) (*Fig.3-3*). This extremly high P concentration could have resulted from the fraction of available P in the NaOH solution with small amout of sample (soil or sediment), leading to asymtotic behaviour. Additionally, at the small values of sample to extractant ratio (less than 0.50 mg mL^{-1}), the P concentration fluctuated. This may have resulted from the greater volume of solution into which the P could diffuse. To avoid this problem, the optimal ratio was chosen as 1.0 mg mL^{-1}, because the extracted P at this point in most cases was the most similar (slope = 0.96, r = 0.98) (*Fig.3-4vi*) to the values obtained using the conventional mechanical shaking method. This is different with the advice of Sharpley *et al.* (1991), who proposed that the ratio as 2.0 mg mL^{-1} was the most effective ratio for chemical extractions.

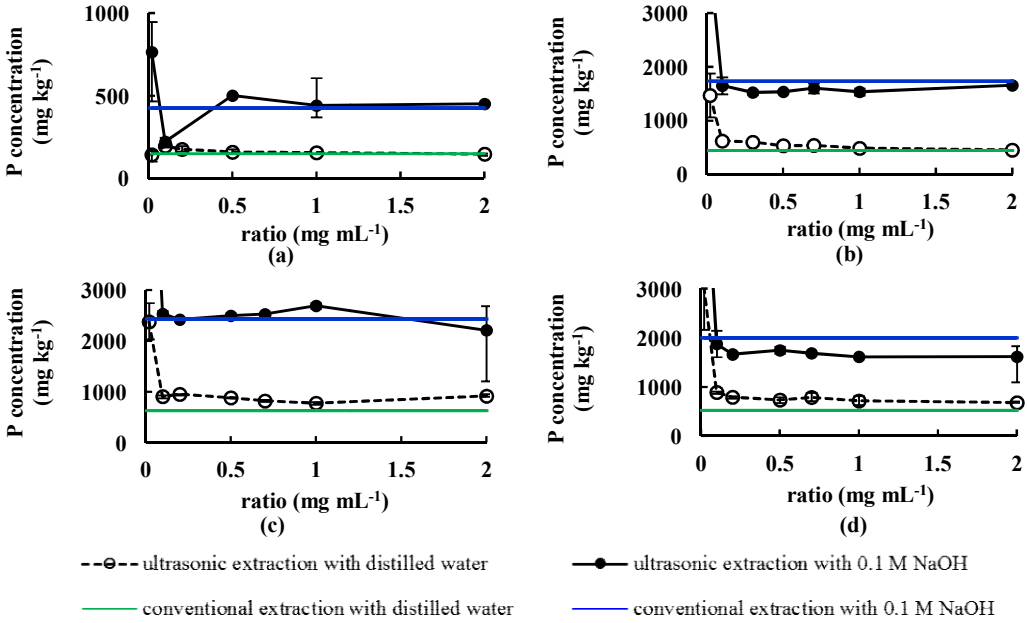

Figure 3-3. Effect of ratio of sample (soil or suspended sediment) to extractant volume on the extracted P. Each value represented the mean of triplicates. Bars represent positive and negative errors.

Based on these results, the effect of sonication time on the amount of extracted P was studied using 30 W and a ratio of 1.0 mg mL^{-1} of soils or sediments to extractant. There was no clear pattern for the relationship between concentration of extracted P and the duration of ultrasonic treatment although there was some evidence that the P concentration increased with increasing extraction duration (*Fig.3-5*). Extending the extraction time beyond 15 min did not increase the efficiency of extraction, thus using a shorter extraction time would improve the working efficiency. *Fig.3-5* shows that the quantity of P extracted in 1 min was similar to the values obtained using other durations, and the values at this duration were generally close to those obtained by the conventional mechanical shaking treatment (intergrating data: slope = 1.01, r = 0.98; 0.1 M NaOH extraction individually: slope = 1.01, r = 0.98) (*Fig.3-6i*).

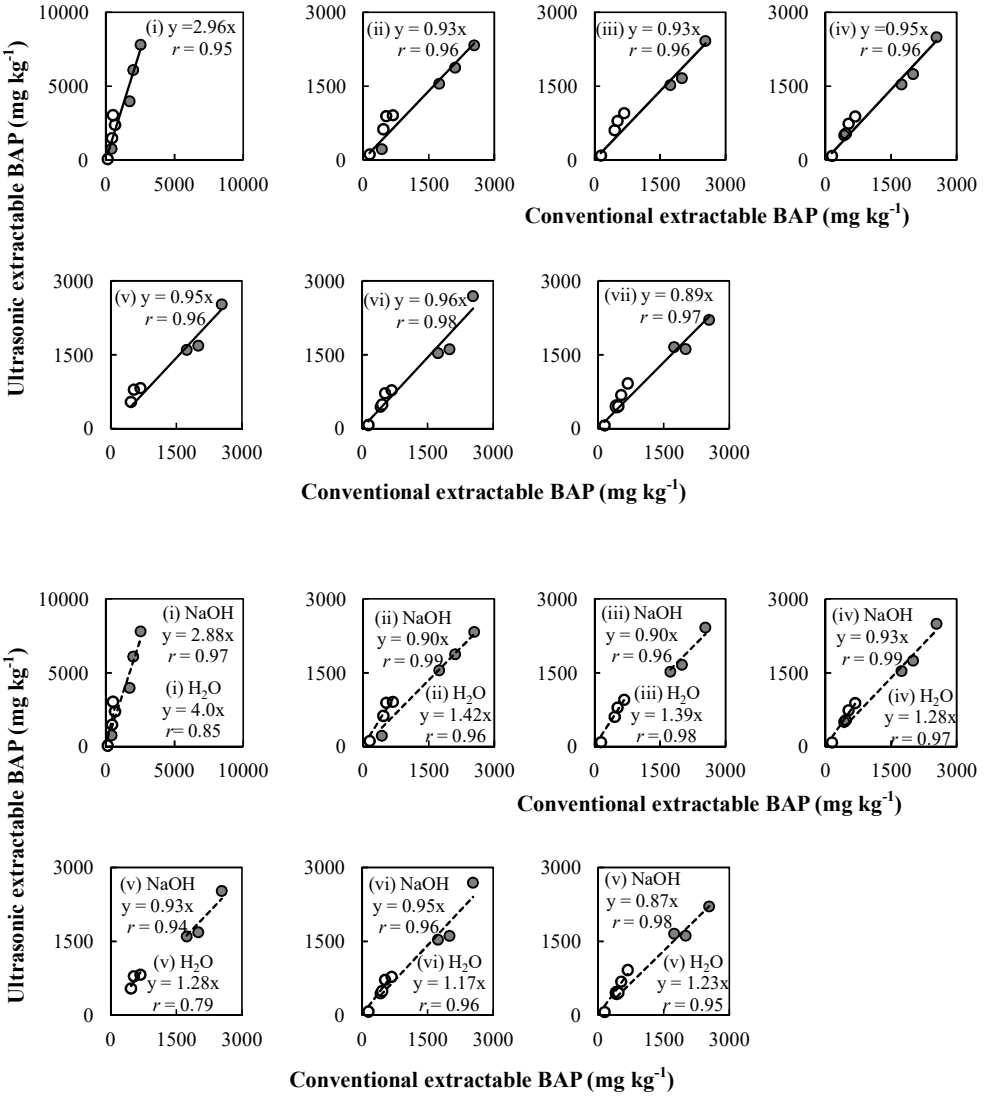

Figure 3-4. Pearson correlations between extraction methods when the ratio was 0.02 (i), 0.1 (ii), 0.2 (iii), 0.5 (iv), 0.7 (v), 1.0 (vi), 2.0 (vii), respectively.

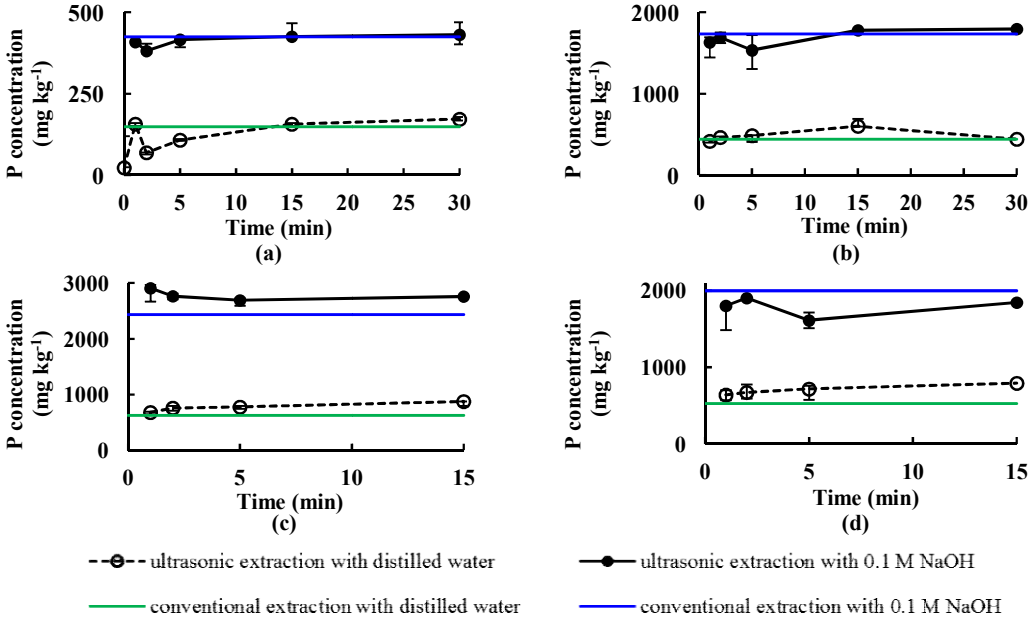

Figure 3-5. Effect of extraction time on the extracted P. Each value represented the mean of triplicates. Bars represent positive and negative errors.

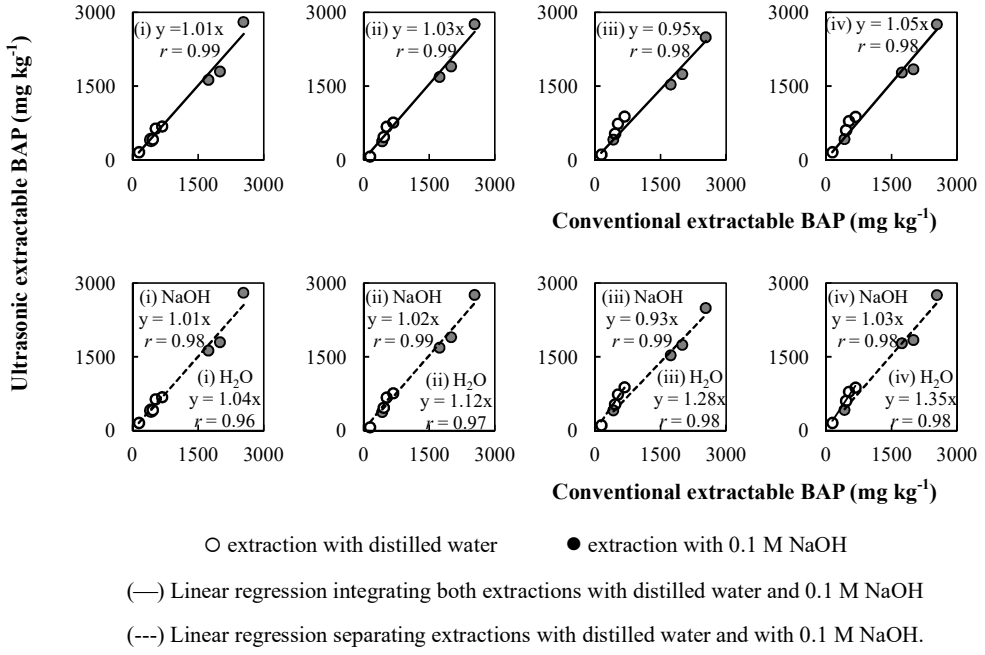

Figure 3-6. Pearson correlations between extraction methods when the extraction time was 1 min (i), 2 min (ii), 5 min (iii), 15 min (iv) respectively.

Therefore, the optimal conditions for extraction with 0.1 M NaOH appear to be 30 W of ultrasonic intensity for 1 min of extraction duration, and a ratio of 1.0 mg sample (soil or sediment) per mL of extractant.

3.4.2 Comparison between the extraction methods

Table 3-1 summarized the concentrations of P obtained by the different extraction methods. On average, the concentrations of P obtained using distilled water extraction under the optimal conditions for the ultrasonic method ranged from 14.0 to 24.8% of total P, versus 13.2 to 20.3% for the conventional method. In the 0.1 M NaOH extraction, P values ranged from 36.4 to 85.3% of TP, versus 38.4 to 77.5% for the conventional method. Our results were in close agreement with those of previous studies which proved percent BAP of TP was 32.0 to 83.0% in runoff (Fabre et al., 1996; Sharpley, 1993). In addition, the P concentrations extracted using 0.1 M NaOH plus ultrasonic treatment were notably ($F = 99.0$, $p < 0.0001$) (*Table 3-3*) higher than those extracted by distilled water. Even after only 1 min, much amount of the total P was extracted by both the mechanical shaking and ultrasonic treatment methods (*Fig.3-5* and *Table 3-1*). There are two likely explanations. First, NaOH is a stronger solvent than distilled water, thus it is able to extract more P. Second, NaOH extraction may lead to selective extraction of iron- bound P that would not be extracted by water (Holtan et al., 1988). The color of sediment samples were yellow (Sediment c and Sediment d) and yellowish grey (Sediment b), possibly due to the presence of iron components. Therefore, the P concentrations obtained by extracting these sediment samples with NaOH would be higher than those obtained from the soil samples which was dark grey and therefore probably contained less iron.

Table 3-1. Mean values of extracted P concentration obtained using the different methods. (a) Soil sample collected on 2011 January 26th; (b) Suspended sediment sample collected on 2014 September 5th; (c) Suspended sediment sample collected on 2014 September 11th; (d) Suspended sediment collected on 2014 October 6th. Each value represented the mean of triplicates (± SD).

P conc. (mg kg^{-1})		Soil (a)	Sediment (b)	Sediment (c)	Sediment (d)
TP		1124 ± 155	2819 ± 128	3413 ± 44	2580 ± 49
Water extraction	Conventional extraction 17h	148 ± 40	444 ± 19	627 ± 47	525 ± 52
	Conventional extraction 1min*	n.a.	130 ± 00	140 ± 00	130 ± 00
	Ultrasonic extraction 5 min	108 ± 50	535 ± 28	883 ± 34	736 ± 10
	Ultrasonic extraction 1 min	157 ± 50	417 ± 58	680 ± 46	639 ± 19
0.1 M NaOH extraction	Conventional extraction 17h	425 ± 15	1736 ± 186	2435 ± 760	2000 ± 41
	Conventional extraction 1min*	n.a.	1080 ± 000	2010 ± 00	1180 ± 00
	Ultrasonic extraction 5 min	416 ± 90	1536 ± 176	2497 ± 878	1748 ± 317
	Ultrasonic extraction 1 min	409 ± 21	1632 ± 300	2908 ± 123	1803 ± 53

* Values of P concentration extracted by shaking in 1 min were measured once.

Table 3-2. ANOVA for the effect of extraction methods (ultrasonic treatment versus conventional extraction, using distilled water versus 0.1 M NaOH) on the concentration of P extracted from four samples.

Source of variation	Degrees of freedom	F-ratio	p-value
Extraction methods	3	99.00	<0.0001
Sample types	3	146.46	<0.0001
Interaction	9	14.07	<0.0001
Total	47		

3.5. Summary

The examination of applying ultrasonic treatment for the extraction of BAP from an agricultural soil and suspended sediments was described. We determined the optimal conditions for the ultrasonic treatment, with distilled water and NaOH as extractants; the NaOH extracted a higher proportion of BAP. The technically simple procedure proposed in this study is an extraction time of only 1 min, compared to 17 hours for BAP extraction using the conventional mechanical shaking method. The ratio of soil to extractant in the new technique is also lower, at 1 mg mL^{-1}. The BAP values obtained using the ultrasonic treatment with 0.1 M NaOH were similar to (r = 0.98) those obtained using the conventional method. These findings suggest that the new method provides a promising alternative for quantifying BAP in soils and river sediments. Further research should be done to test the "real" bioavailability for algal growth of BAP obtained from the extractions.

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Chapter 4 Algal Growth Potential Indicators of Particulate Phosphorus After NaOH Extraction

4.1. Outlines

The purpose of this chapter was to preliminarily evaluate the bioavailability for algal growth of particulate BAP which was estimated in soil and suspended sediment samples by the extractions proposed in the previous chapter. The evaluation was conducted by applying the residues after NaOH extractions to AGP tests. The 0.1 M NaOH was assumed to be able to entirely extract BAP in a single treatment, i.e. the algae would not grow in the media incubated with sample residues after extractions due to lack of P supply. The samples without any extraction were defined as "control sample" (I); the samples extracted with ultrasonic treatment were termed extracted samples "with ultrasonic treatment" (II); and the samples extracted by conventional mechanical shaking for 17 hours were called "with mechanical shaking" (III). P-free CB media containing no external P source were prepared for "negative controls" (IV), whereas CB-media containing β-glycerophosphate were used for the "positive controls" (V).

4.2. Investigation procedure

2 mL of P-starved *M.aeruginosa* (1.3×10^5 cells mL^{-1}) from the stock solution was cultured into 100 mL Erlenmeyer flask containing 58 mL of P-free CB media and dried samples (respectively I, II and III), equivalent to 0.2 mg-P L^{-1}, as the sole P source, or negative and positive controls (respectively IV and V). The amount of P supply for algal cultures corresponded to the Chl-*a* in an eutrophic lakes in summer (French and Petticrew, 2007) that should be managed less than 200 μg L^{-1} (Kasprzak et al., 2008).

Incubation occurred in a growth chamber under cool white fluorescent light at around 20 μmol m^{-2} s^{-1} with a 12:12-h light: dark cycle at 25 ºC for 28 days. Growth yield was monitored by indicators such as Chl-*a*, DP, PP, POC and PON in each medium on days 0, 7, 14, 21, 28. All measurements were the average of three independent replicates. The pH in each medium was monitored every week and maintained at 8.5 to 9 by NaOH (1 M) and HCl (1 M). The cell count was observed under microscope to confirm the contamination of indigenous bacteria during incubation period.

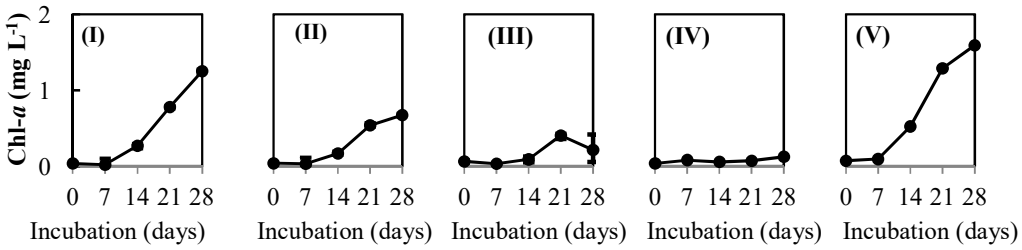
4.3. Samples

The proposed extraction on two samples, including one soil and one suspended sediments: (a) Soil sample collected on 2011 January 26th; (b) Suspended sediment sample collected on 2014 September 5th. Extractions were conducted triplicates. Algal growth potential tests were also examined on triplicate for each sample.

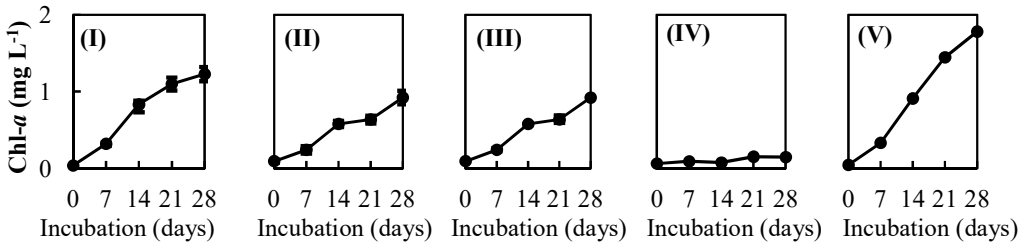
4.4. Results and discussion

Due to the differences among the P contents of the samples, the amounts of soil and sediment required to produce an equivalent concentration of 0.2 mg-P L^{-1} differed between the media. The amounts of soil added to the media were 12.0 (I), 19.6 (II), and 20.9 (III) mg, whereas the amounts of sediments were 3.5 (I), 8.8 (II), and 8.7 (III) mg.

Fig.4-1 illustrated the concentration of Chl-*a* over incubation time. In all media containing samples, the Chl-*a* increased (*Fig. 4-1 I, II, III*) but lower than the values in media containing *β*-glycerophosphate (*Fig. 4-1 V*). This result implied that *M. aeruginosa* could grow when samples (soil or sediment) presented as a sole P-source in each medium. It suggested *M.aeruginosa* could uptake some of P in particulate forms



(a) Soil samples



(b) Sediment samples

Figure 4-1. Chl-*a* concentrations in cultures of *M. aeruginosa.* (I) Control samples, (II) sample extracted with ultrasonic treatment, (III) sample extracted with the conventional method, (IV) no source of P (negative control), (V) *β*-glycerophosphate (positive control). Each value is the mean of triplicates. Bars represent positive and negative errors.

which were remained after the extraction and considered to contain insignificant BAP. The dramatic growth of Chl-*a* concentrations in positive control media confirmed that *M.aeruginos*a grew healthily in well P supply (*Fig. 4-1 V*). The weaker rise of Chl-*a* in the media containing samples after extraction (*Fig.4-1 II, III*) than in those containing control samples (*Fig.4-1 I*) proved that the extraction had successfully obtained partial BAP fraction from samples. Additionally, Chl-*a* concentrations increased insignificantly in negative control media (*Fig.4-1 IV*) reinforced that *M. aeruginosa* did not grow significantly in the P-free environment.

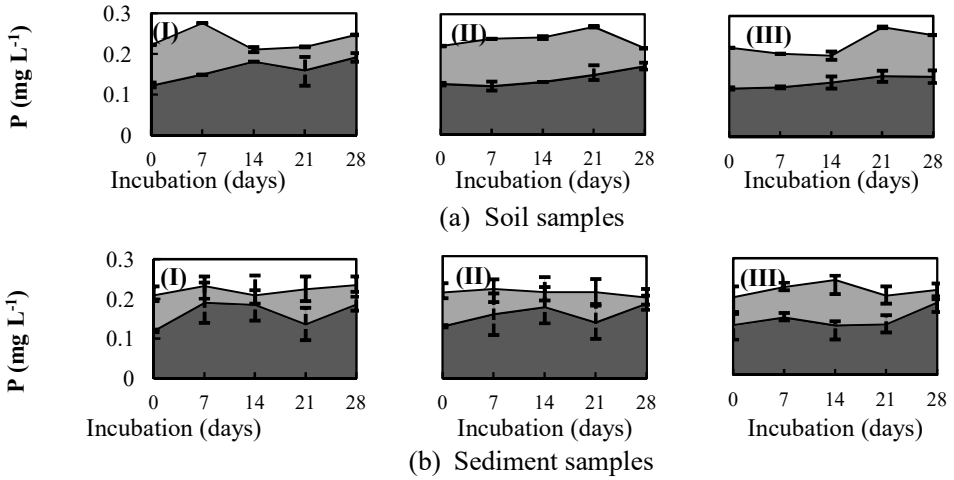


Figure 4-2. Concentrations of (■) PP and (■) DP (left) in cultures of *M. aeruginosa*. (I).Control sample, (II) samples extracted with ultrasonic treatment, (III) samples extracted with the conventional method. Each value is the mean of triplicates. Bars represent positive and negative errors.

Fig.4-2 compared the concentrations of PP and DP in each medium over the incubation. The increase of Chl-*a* in media containing samples as P-source resulted in the rise of Chl-*a* to PP ratio (*Fig. 4-3*). It was quite different as we expected that PP would be remain stable in all media containing samples whereas Chl-*a* would not go up in the media containing samples after extraction. Although there has been no clear pattern for the relationship between the cell growth (Chl-*a*) and the increase of PP in each medium, the ratio values of Chl-*a* to PP ranged 0.3 to 20.5 in cultures incubated with all three sample types (*Fig. 4-3*) is consistent with study of Spears et al. (2013). It reported if the ratio of Chl-*a* to PP more than 0.2, P could be an important factor limiting algal growth. The fluctuation of P concentration during incubation (*Fig. 4-2*) would be able to be controlled if we could separate the algal cells from the particulate matters (soil or sediment).

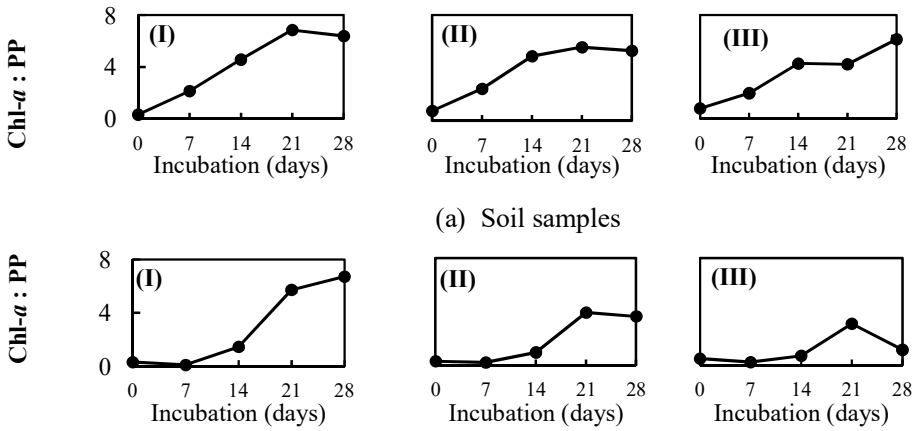


Figure 4-3. Ratios of Chl-*a* to PP in cultures of *M. aeruginosa*. (I).Control sample, (II) samples extracted with ultrasonic treatment, (III) samples extracted with the conventional method. Each value is the mean of triplicates. Bars represent positive and negative errors.

The percentages of POC and PON to SS in all media were relatively stable despite of the rise of Chl-a (*Fig.4-4 I, II, III*). The presence of POC and PON in *Fig. 4-4 IV* was assigned to the intracellular P in *M.aeruginosa*'s cells. The difference between the upper and lower *Fig. 4-4.IV* was allergered by the difference of the initial population of the cultured algae. The dramatic increase of POC concentrations in the media of positive controls (*Fig. 4-4 V*) illustrated the healthy growth of *M.aeruginosa* due to the presence of β-glycerophosphate. The C/N ratios increased over incubation time and varied from 4.29 to 8.72 in the media containing samples as the sole-P source. The rise of C/N ratios indicated carbohydrates were higher in P limited cells than in control cells of *M.aeruginosa*.

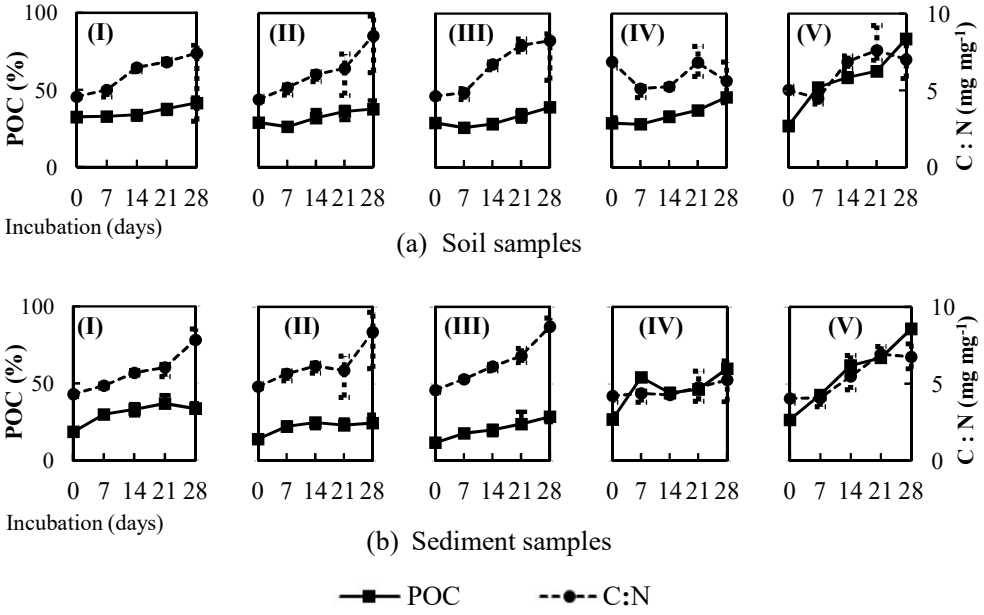
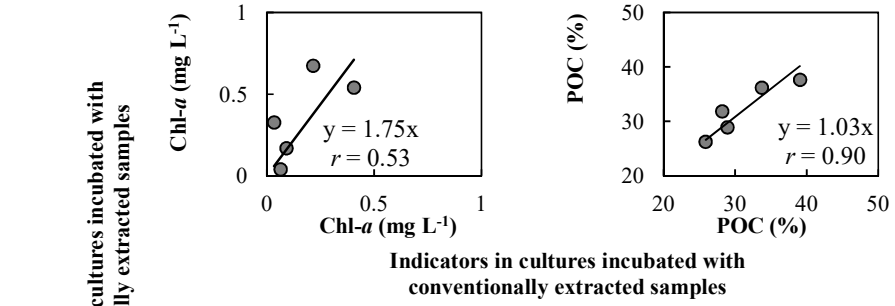


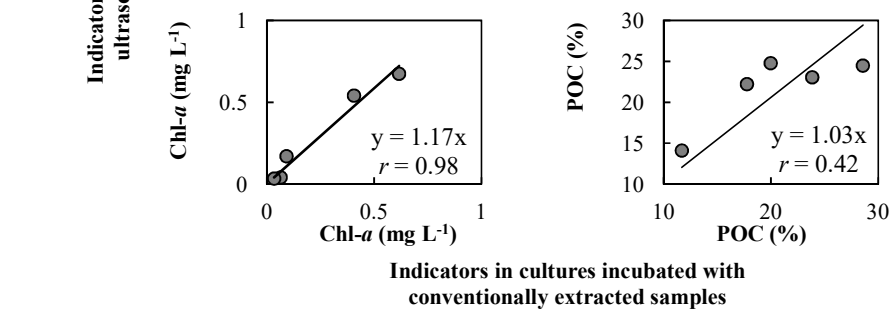
Figure 4-4. Concentrations of POC and ratios of POC to PON in cultures of *M. aeruginosa.* (I) Control sample, (II) sample extracted with ultrasonic treatment, (III) sample extracted with the conventional method, (IV) no source of P (negative control), (V) β-glycerophosphate (positive control). Each value is the mean of triplicates. Bars represent positive and negative errors.

In the other words, the algae grew under P-limited conditions (Okubo et al., 2014). It reinforced the aforementioned argument that P could be the limiting factor of algal growth. The observation under microscope detected no indigenous bacteria during the incubation period.

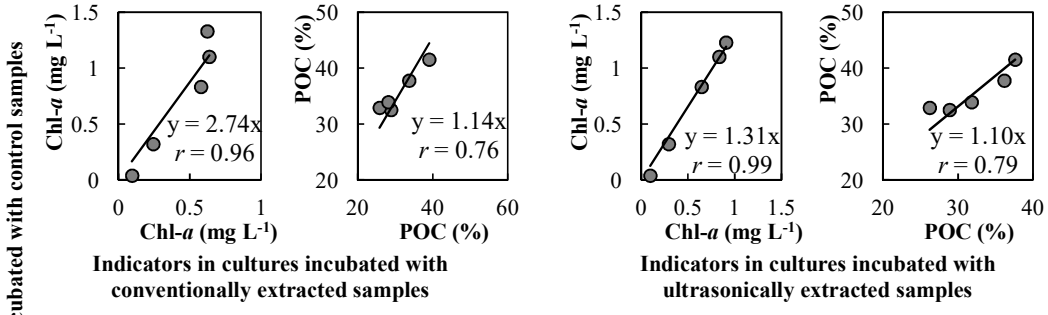
Fig.4-5 showed the comparison of growing indicators in cultures incubated with the ultrasonically extracted samples and in those incubated with the conventionally extracted samples. The slopes were 1.03 to 1.75 illustrated that the growth of *M. aeruginosa* was similar in media containing extracts of soil or sediment obtained with ultrasonic treatment and with mechanical shaking.



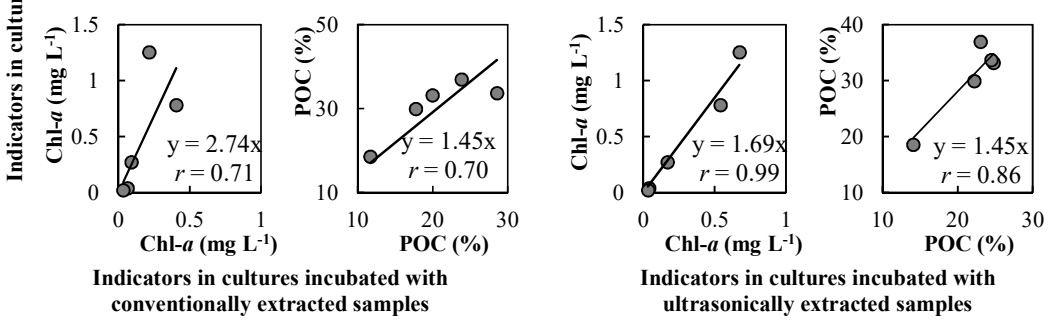
(a) Soil samples



(b) Sediment samples



(a) Soil samples



(b) Sediment samples

Figure 4-5. Similarity of indicators in cultures incubated with conventionally extracted samples and with ultrasonically extracted samples. Comparison with control samples.

4.5. Summary

M. aeruginosa failed to grow in the negative control medium that contained no soil, sediment, β-glycerophosphate, or any other source of P. The fact that *M. aeruginosa* grew in a medium containing extracted soil or sediment implies that this alga can use intracellular forms of P for their growth or some forms of P that remain after extraction. The latter have been considered to be non-bioavailable. Further study should clarify the P species that remain after extraction. A technique to separate the intracellular forms of P in *M. aeruginosa* from the particles in soil is also necessary. However, *M. aeruginosa* grew much better in a medium containing β-glycerophosphate than in media containing soil or sediment particles. The growth of *M. aeruginosa* was similar in media containing extracts of soil or sediment obtained with ultrasonic treatment and with mechanical shaking. Furthermore, the ratios of BAP to TP in samples extracted using ultrasonic treatment with 0.1 M NaOH were similar to those extracted using the conventional method. These results suggested that the extraction time can be shortened from 17 hours with the conventional method to 1 min.

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Chapter 5 Bioavailability of P Extracted from Soils and Suspended Sediments

5.1. Outlines

In previous chapters, we successfully figured out the optimal working conditions of ultrasonic extraction and the suitable environment for algal growth potential test subjected to soil and one sediment sample. Therefore, the main purpose of this chapter was to verify the bioavailability of P in a variety of soil and sediment samples, and the relationship between the P obtained from extractions and the algal growth.

5.2. Investigation procedure

BAP concentration in each sample (soil and suspended sediment) was estimated by means of a single-step extraction (conventional mechanical shaking or proposed ultrasonic method) as described in Chapter 2 and 3. To evaluate extraction efficiency, we calculated recovery rate by dividing the sum of the P in the supernatants and the P in the solid residues by the total amount of P in the original samples of soil and sediment before extraction. In order to prepare for the bioassays, the residues remaining after either mechanical shaking or ultrasonic extraction and subsequent centrifugation were washed twice with distilled water to remove any remaining NaOH, and the washed residues were air-dried at 40 ^{O}C.

Three types of samples were subjected to AGP bioassays: unextracted soil and sediment samples (control samples, designated as “I”) and solid residues obtained either by NaOH extraction of soil and sediment samples with mechanical shaking for 17 h (designated as “II” or “mechanically extracted samples”) or by NaOH extraction of soil

and sediment samples with ultrasonication (designated as "III" or "ultrasonically extracted samples").

P-starved *M. aeruginosa* (1.3×10^5 cells mL^{-1}) was incubated in 200 mL of P-free CB medium containing each of the samples described in the preceding paragraph as the sole P source, in an amount equivalent to 0.1 mg-P L^{-1} (van Puijenbroek et al., 2014). The optimal pH for growth of *M. aeruginosa* is approximately 9, so the pH was monitored and maintained at that value during the incubation (Kasai et al., 2004). Note that these AGP bioassays were conducted indirectly—that is, with the residues left after extraction and centrifugation of soil and sediment samples as the P source, rather than direct way with the extracts themselves—because the extracts contained too much NaOH (pH > 12) and the salinity of neutralized aliquots was too high (>5%) for *M. aeruginosa* growth.

As a negative control, algae were incubated in P-free CB medium containing no external P source; and as a positive control, algae were incubated in CB-medium containing *β*-glycerophosphate. In all cases, incubation was carried out at 25 ^{O}C for 42 days in a growth chamber under cool white fluorescent light with a 12:12-h light: dark cycle at 20 μmol m^{-2} s^{-1}. Microscopic cell counts were determined with a hemocytometer to monitor for contamination by indigenous bacteria during the incubation period.

The concentrations of chlorophyll-*a* (Chl-*a*), particulate organic carbon (POC), particulate organic nitrogen (PON), DP, and PP were used as indicators of algal growth. Concentrations were measured for samples collected on incubation days 0, 7, 25, and 42 and are reported as averages of three independent replicates per sample. The concentrations of the indicators in the cultures incubated with investigating particulate

samples (soil and sediment) are obtained by subtracting the blank values for the negative controls from the means of triplicate values of each sample. The cell count was observed under microscope to confirm the contamination of indigenous bacteria during incubation period.

5.3. Samples

The proposed extraction was tested on all of four samples, including soil (a), sediment (b), sediment (c), sediment (d) (described in Chapter 2). AGP bioassays subjected to three types of samples, including control samples (I), conventional mechanically extracted samples (II), proposed ultrasonic extracted samples (III). Therefore, we got the below summary list of samples which was applied to AGP bioassays (*Table 5-1*). Extractions were conducted triplicates. Algal growth potential tests were also examined on triplicate for each sample. P concentrations were the average of duplicate analyses.

5.4. Results and discussion

5.4.1 Quantification of extractable BAP

The amounts of BAP extracted by the ultrasonic extraction method were similar to the amounts extracted by the conventional mechanical extraction method (*Fig. 5-1, 5-2*). The proportions of extractable BAP in the soil a, sediment b, sediment c, and sediment d samples were 34.2%, 35.5%, 55.2%, 45.8% , respectively, when we used the ultrasonic extraction method; and the corresponding values for the conventional method were 38.9%, 39.1%, 48.9%, 40.6% (*Fig. 5-1*). All these percentages are consistent with values determined in a previous study indicating that the proportion of BAP in PP in agricultural streams ranges from <5% to 69%, depending on the type of agriculture in

the watershed of the stream (Ellison and Brett, 2006).

Table 5-1. The samples subjected to AGP bioassays

ID	Sample name	Definition
aI	Control soil	Original soil before extraction
bI	Control sediment (b)	Original sediment (2014 Sep. 5) before extraction
cI	Control sediment (c)	Original sediment (2014 Sep. 11) before extraction
dI	Control sediment (d)	Original sediment (2014 Oct. 6) before extraction
aII	Conventional mechanically extracted soil	Solid residue obtained by NaOH extraction of soil with mechanically shaking for 17h
bII	Conventional mechanically extracted sediment (b)	Solid residue obtained by NaOH extraction of sediment (2014 Sep.5) with mechanically shaking for 17h
cII	Conventional mechanically extracted sediment (c)	Solid residue obtained by NaOH extraction of sediment (2014 Sep.11) with mechanically shaking for 17h
dII	Conventional mechanically extracted sediment (d)	Solid residue obtained by NaOH extraction of sediment (2014 Oct. 6) with mechanically shaking for 17h
aIII	Proposed ultrasonically extracted soil (a)	Solid residue obtained by NaOH extraction of soil with ultrasonication
bIII	Proposed ultrasonically extracted sediment (b)	Solid residue obtained by NaOH extraction of sediment (2014 Sep.5) with ultrasonication
cIII	Proposed ultrasonically extracted sediment (c)	Solid residue obtained by NaOH extraction of sediment (2014 Sep.11) with ultrasonication
dIII	Proposed ultrasonically extracted sediment (d)	Solid residue obtained by NaOH extraction of sediment (2014 Oct. 6) with ultrasonication

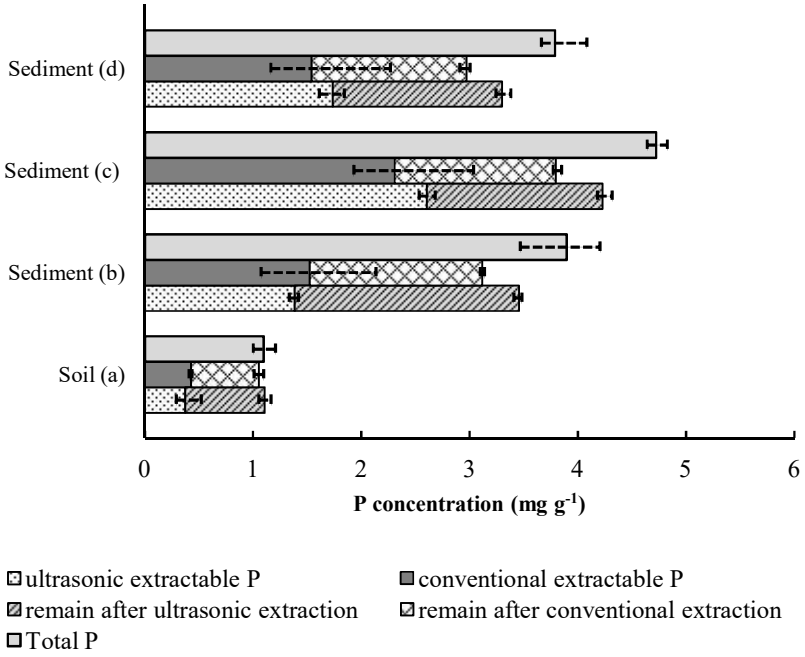

Figure 5-1. Concentrations of extractable phosphorus obtained by ultrasonic extraction and conventional mechanical extraction, along with total P concentrations. The designations a–d correspond to soil samples collected on 2011 January 26 and suspended sediment samples collected on 2014 September 5, September 11, and October 6, respectively. Concentrations are means of triplicate values; bars represent positive and negative errors.

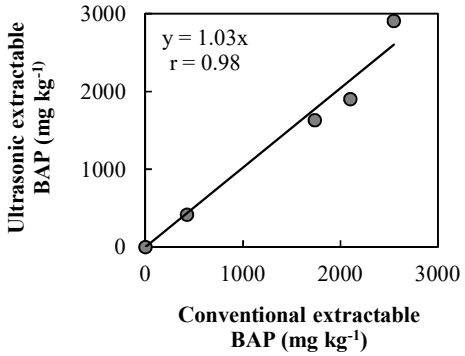

Figure 5-2. Correlation between concentrations of extractable phosphorus obtained by ultrasonic extraction and by conventional mechanical extraction.

Our results also confirm that 0.1 M NaOH is an appropriate solvent for BAP extraction (Sharpley et al., 1991, Dorich et al., 1985): the average P recovery rates for the mechanical and ultrasonic extraction methods were high: 96.1% for soil I, 80.0% for sediment II, 80.5% for sediment III, and 78.4% for sediment IV for conventional mechanical extraction and 99.9% for soil I, 88.6% for sediment II, 89.5% for sediment III, and 87.1% for sediment IV for ultrasonic extraction. In addition, the similarities between the two sets of recoveries suggest that ultrasonic extraction with 0.1 M NaOH is an appropriate method for estimating BAP.

5.4.2 Algal available P

We cultured P-starved *M. aeruginosa* (1.3×10^5 cells mL^{-1}) in P-free CB medium containing soil samples aI–III and sediment samples bI–III, cI–III, and dI–III as the sole P source in an amount equivalent to 0.1 mg-P L^{-1}, which is the approximate TP concentration that limits algal growth as stipulated in environmental quality standards (van Puijenbroek et al., 2014). Because the TP concentrations in the samples differed (*Fig. 5-1*), the average amounts of the samples included in 200 mL of P-free CB medium were as follows: 18.3 mg of Soil aI, 27.3 mg of Soil aII, 31.9 mg of Soil aIII, 5.1 mg of Sediment bI, 9.7 mg of Sediment bII, 12.5 mg of Sediment bIII, 4.2 mg of Sediment cI, 12.3 mg of Sediment cII, 13.4 mg of Sediment cIII, 5.3 mg of Sediment dI, 12.8 mg of Sediment dII, and 13.9 mg of Sediment dIII.

Observation under a microscope revealed that there were no indigenous bacteria in any of the culture media during the incubation period. It implies there was no interference of indigenous bacteria on *M.aeruginosa*'s growth during AGP bioassays.

The results obtained for the negative control cultures show that even when there was no

external source of P, Chl-*a* concentration increased slightly until day 25 of the incubation period (*Fig. 5-3*). This result suggests that *M. aeruginosa* could grow in a P-depleted environment by using the limited amount of P present in the algal cells. It might be possible to confirm this possibility if the P forms that remaining in samples and extraction residues at the end of the AGP bioassays could be identified. However, currently available techniques (Xu et al., 2010, Dunkley et al., 2008) do not allow for good separation of *M. aeruginosa* cells from soil and sediment particles. For this purpose, a technique for separating algal cells from particulate matter derived from soil or suspended sediment at the end of the bioassays is required.

The temporal dependences of Chl-*a*, POC, and PON concentrations over the entire incubation period for all the samples are shown in (*Figs. 5-4, 5-6, 5-8*), respectively. The concentrations were subtraction of the blank values in the negative controls (*Fig. 5-2*) from the means of triplicate values in samples. The Chl-*a*, POC, and PON curves indicate that the algae grew slowly during the first week and then increased dramatically, peaking on day 25 and then declining to the death phase at around 40 days. These results confirm previous results indicating that *M. aeruginosa* reaches the stationary phase 2–3 weeks after the start of incubation (Okubo et al., 2012). The increases in the algal growth indicators (Chl-*a*, POC, and PON concentrations) for samples obtained by mechanical and ultrasonic extraction suggest that *M. aeruginosa* could take up some of the PP that remained after extraction and that was considered to contain insignificant amounts of BAP. These results appear to contradict our hypothesis that the entire BAP fraction could be obtained by means of a single extraction, that is, that no BAP would remain in the residues after mechanical or ultrasonic extraction with NaOH and that the algae would thus not grow when incubated with the extraction residues as the sole P

source.

However, the fact that the increases in the Chl-*a*, POC, and PON concentrations in culture media containing extraction residues (*Figs. 5-4, 5-6, 5-8 II* and *III*) were smaller than the increases for cultures containing control samples (that is, unextracted samples; *Figs. 5-4, 5-6, 5-8 I*) proved that extraction did remove a substantial proportion of the BAP from the soils and sediments. Furthermore, the increases in the Chl-*a*, POC, and PON concentrations in culture media containing solid residues of ultrasonic extraction (*Figs. 5-4, 5-6, 5-8 II*) were similar (*Figs. 5-5, 5-7, 5-9*) to those in culture media containing solid residues of mechanical extraction (*Figs. 5-4, 5-6, 5-8 III*). These results are compatible with the aforementioned BAP quantification results and imply that ultrasonic extraction is a valid alternative method for estimating BAP in particulate samples. However, it was necessary for us to determine whether the P remaining in the solid residues contained BAP that caused algal growth in the AGP bioassays.

We found that the DP and PP concentrations fluctuated over the incubation period, but in most cases the sum of the two concentrations in each medium remained at approximately 0.1 mg L^{-1} (*Fig. 5-10*). Because soil or suspended sediment or solid residue was the sole source of P in each medium, *M. aeruginosa* must have taken up P from the soil or sediment into their cells, which is why PP was the predominant form of P in the bioassay culture medium. Algae grew better in positive control cultures containing dissolved β-glycerophosphate as the sole P source at a concentration of 0.1 mg-P L^{-1} (*Fig. 5-2*) than in media containing samples (*Figs. 5-4, 5-6, 5-8*), even though the sum of the DP and PP concentrations was roughly 0.1 mg L^{-1} in the latter case. This result indicates the amount of the BAP fraction of the PP in the soil and suspended sediment was smaller than the amount of DP in the positive control cultures.

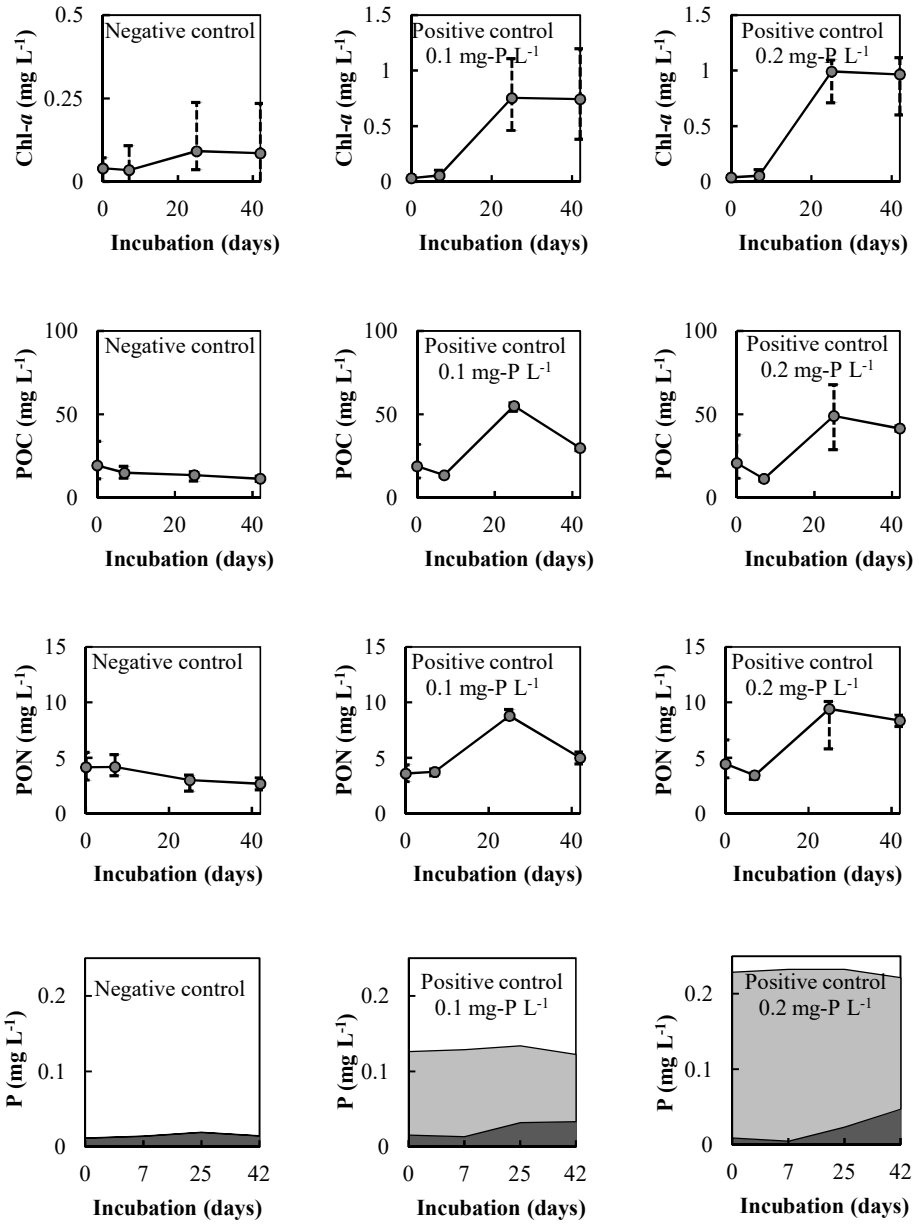

Figure 5-3. Chl-*a*, POC, PON, and DP+PP concentrations in cultures of *M. aeruginosa* incubated with no external P source (negative control), 0.1 mg L^{-1} *β*-glycerophosphate as the sole P source (positive control), and 0.2 mg L^{-1} *β*-glycerophosphate as the sole P source (positive control). Concentrations are means of triplicate values; bars indicate possitive and negative errors.

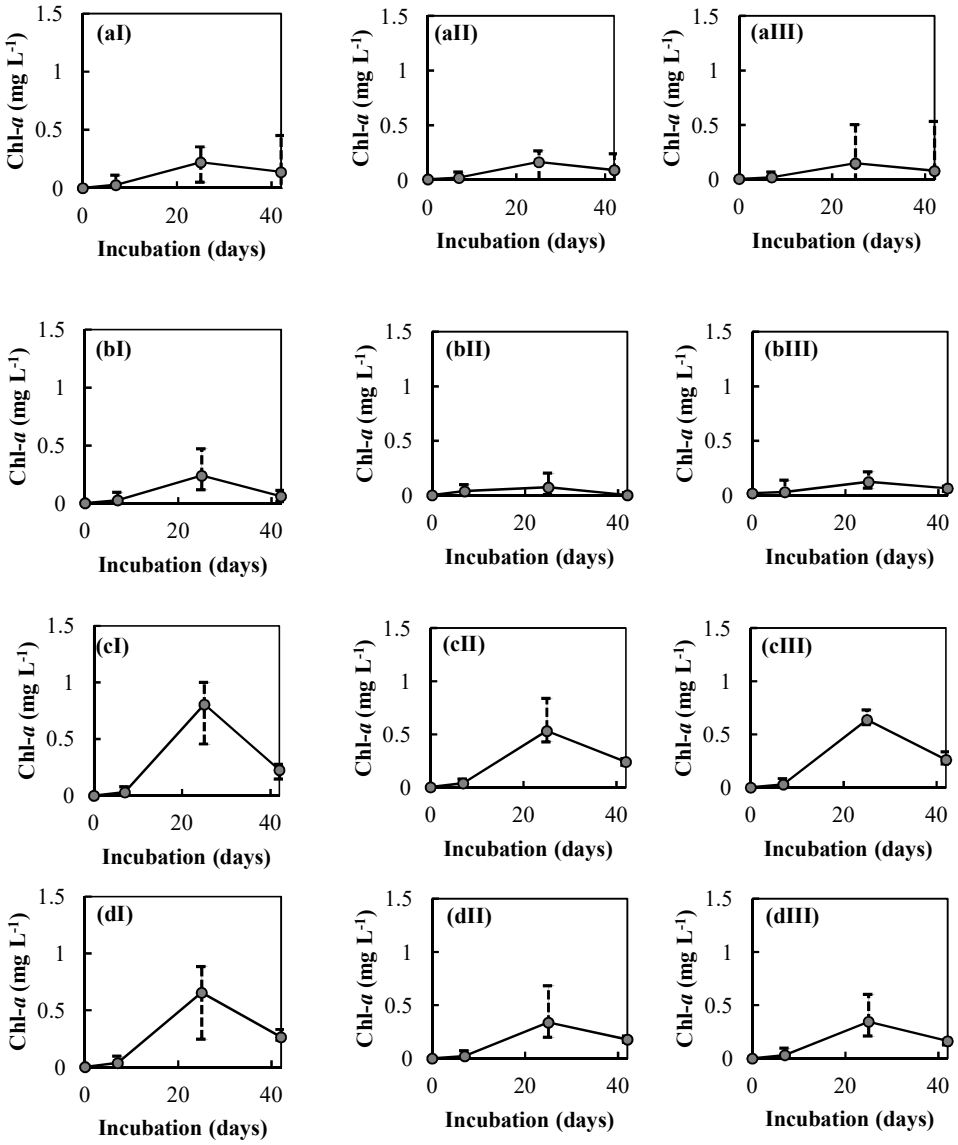

Figure 5-4. Chl-*a* concentrations in *M. aeruginosa* cultures incubated with control samples (I), ultrasonically extracted samples (II), and mechanically extracted samples (III). The designations a–d correspond to soil samples collected on 2011 January 26 and suspended sediment samples collected on 2014 September 5, September 11, and October 6, respectively. Concentrations are means of triplicate values obtained by subtracting the blank values in the negative controls; bars indicate possitive and negative errors.

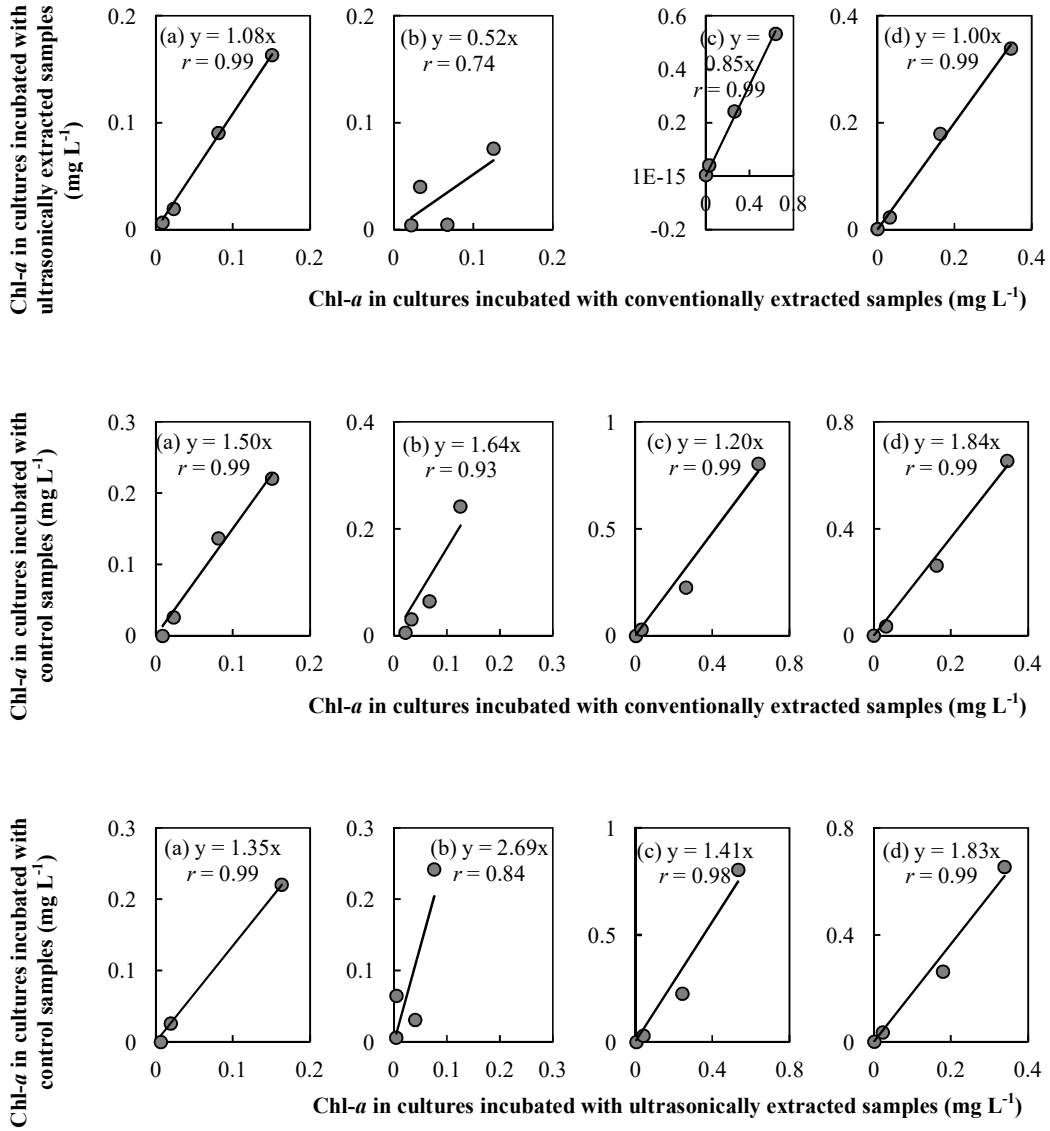

Figure 5-5. Similarity of Chl-*a* concentrations in cultures incubated with conventionally extracted samples and with ultrasonically extracted samples. Comparison with the Chl-*a* concentrations in cultures incubated with control samples. The designations a–d correspond to soil samples collected on 2011 January 26 and suspended sediment samples collected on 2014 September 5, September 11, and October 6, respectively.

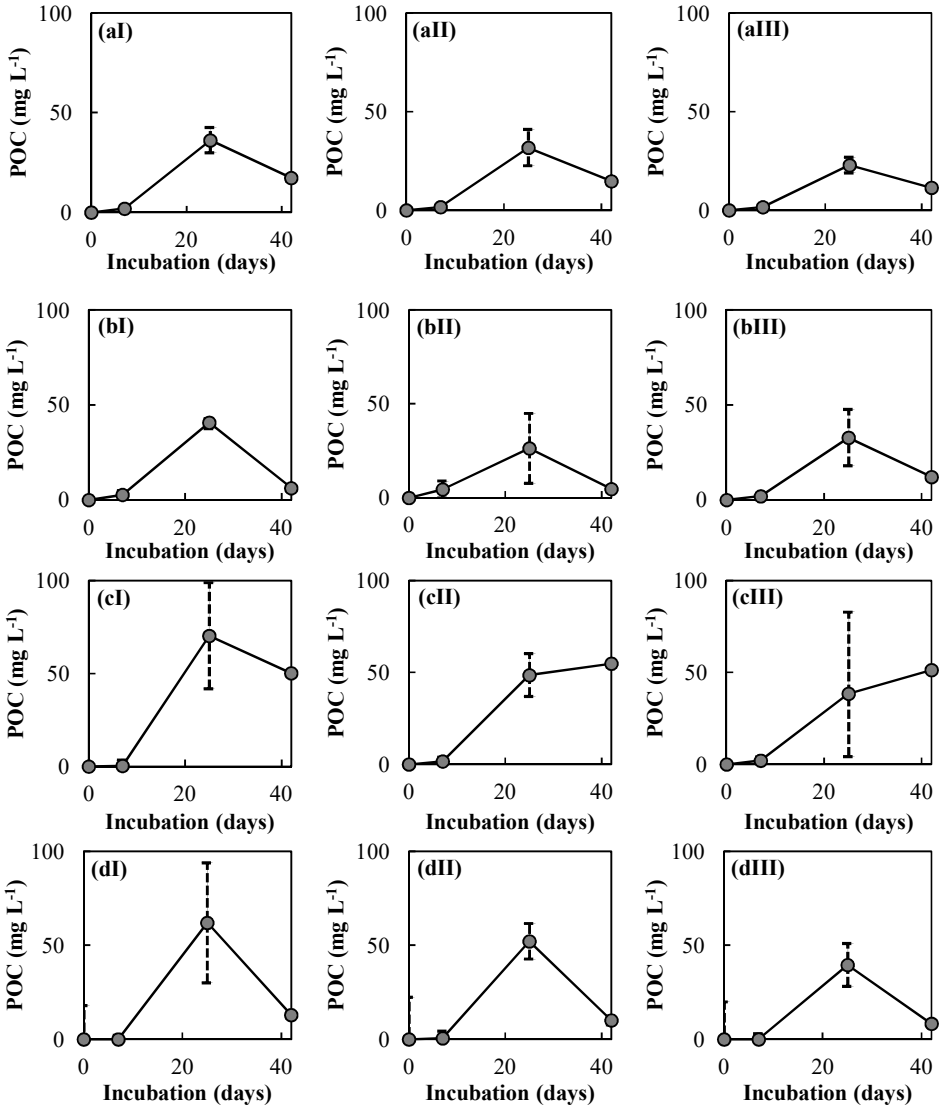

Figure 5-6. POC concentrations in cultures of *M. aeruginosa* incubated with control samples (I), ultrasonically extracted samples (II), and mechanically extracted samples (III). The designations a–d correspond to soil samples collected on 2011 January 26 and suspended sediment samples collected on 2014 September 5, September 11, and October 6, respectively. Concentrations are means of triplicate values obtained by subtracting the blank values in the negative controls; bars indicate possitive and negative errors.

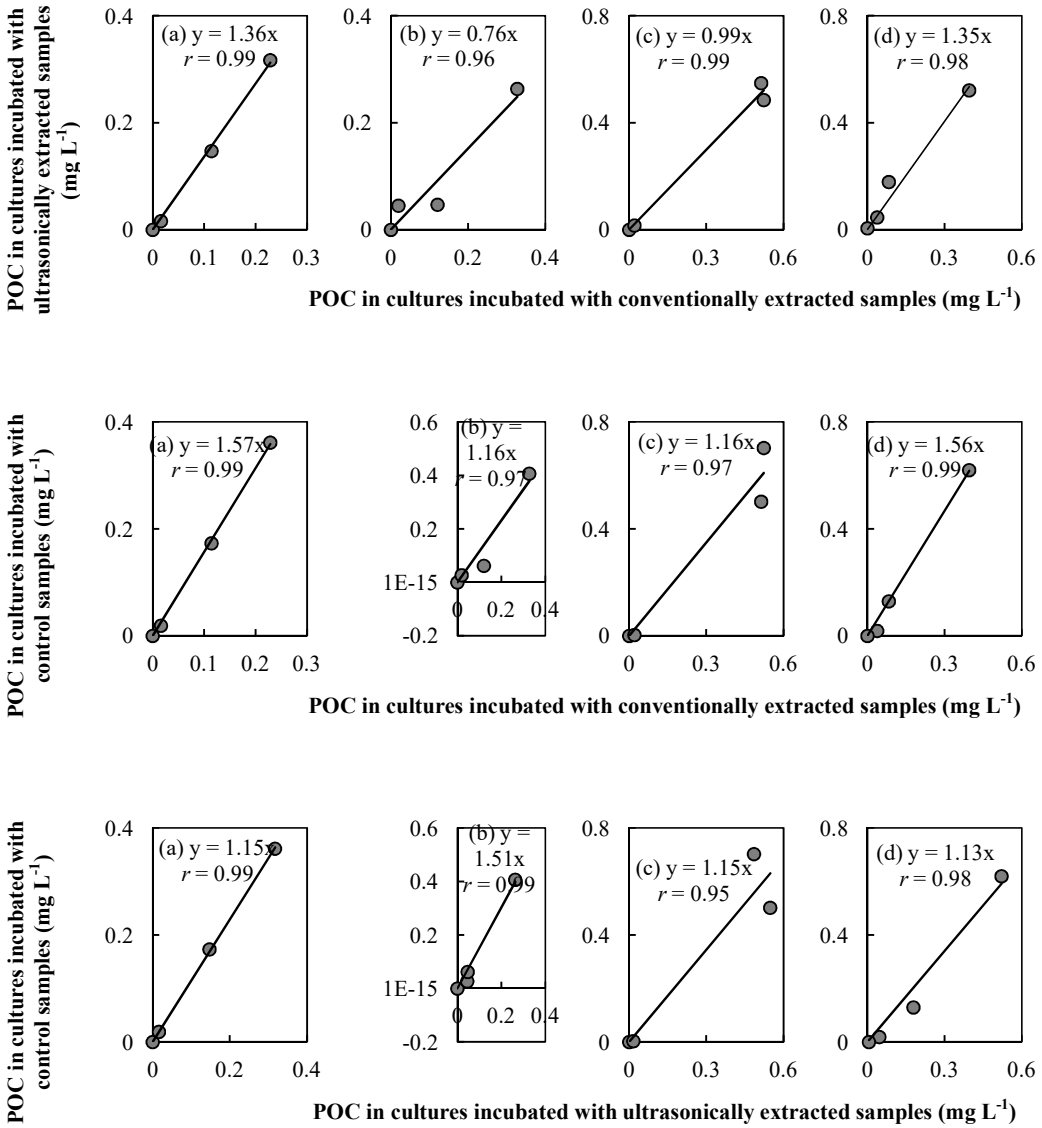

Figure 5-7. Similarity of POC concentrations in cultures incubated with conventionally extracted samples and with ultrasonically extracted samples. Comparison with the POC concentrations in cultures incubated with control samples. The designations a–d correspond to soil samples collected on 2011 January 26 and suspended sediment samples collected on 2014 September 5, September 11, and October 6, respectively.

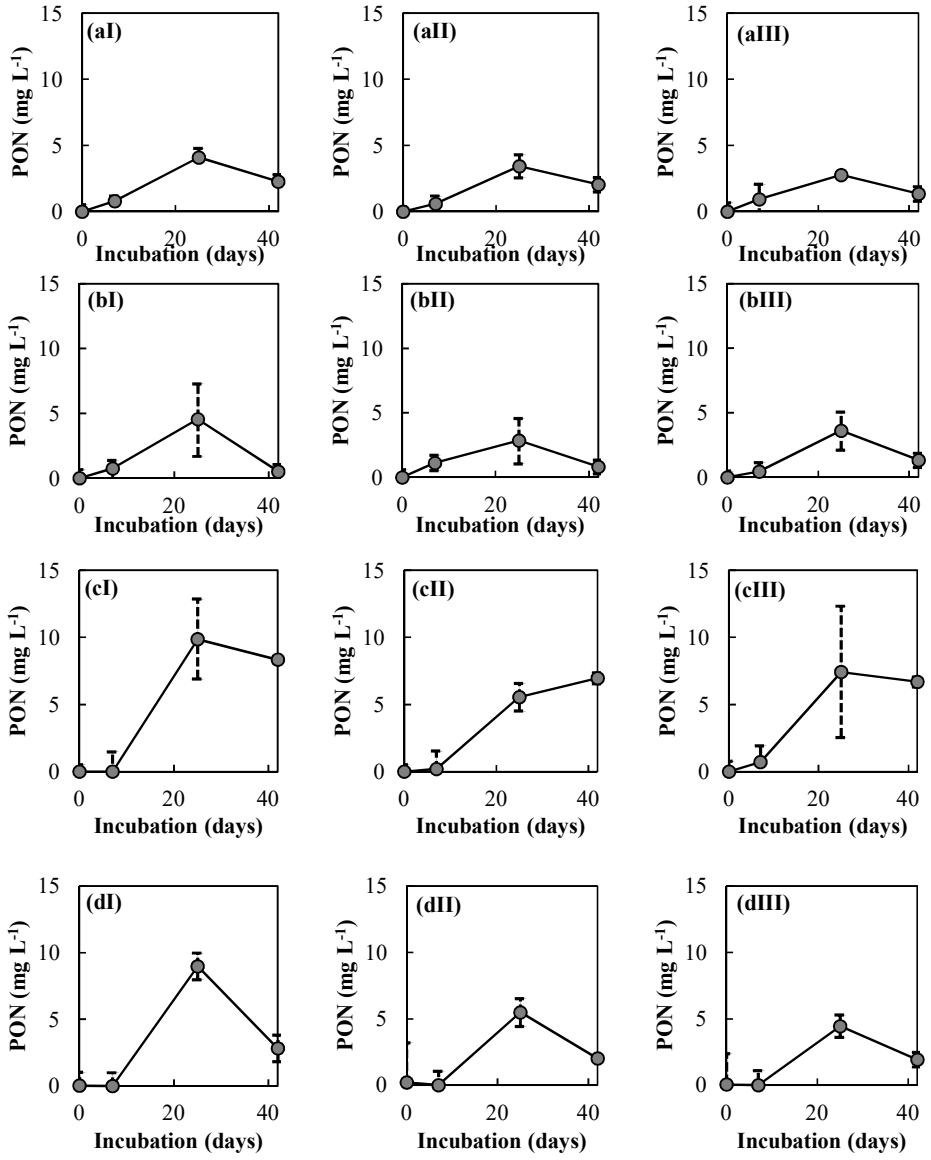

Figure 5-8. PON concentrations in cultures of *M. aeruginosa* incubated with control samples (I), ultrasonically extracted samples (II), and mechanically extracted samples (III). The designations a–d correspond to soil samples collected on 2011 January 26 and suspended sediment samples collected on 2014 September 5, September 11, and October 6, respectively. Concentrations are means of triplicate values obtained by subtracting the blank values in the negative controls; bars indicate possitive and negative errors.

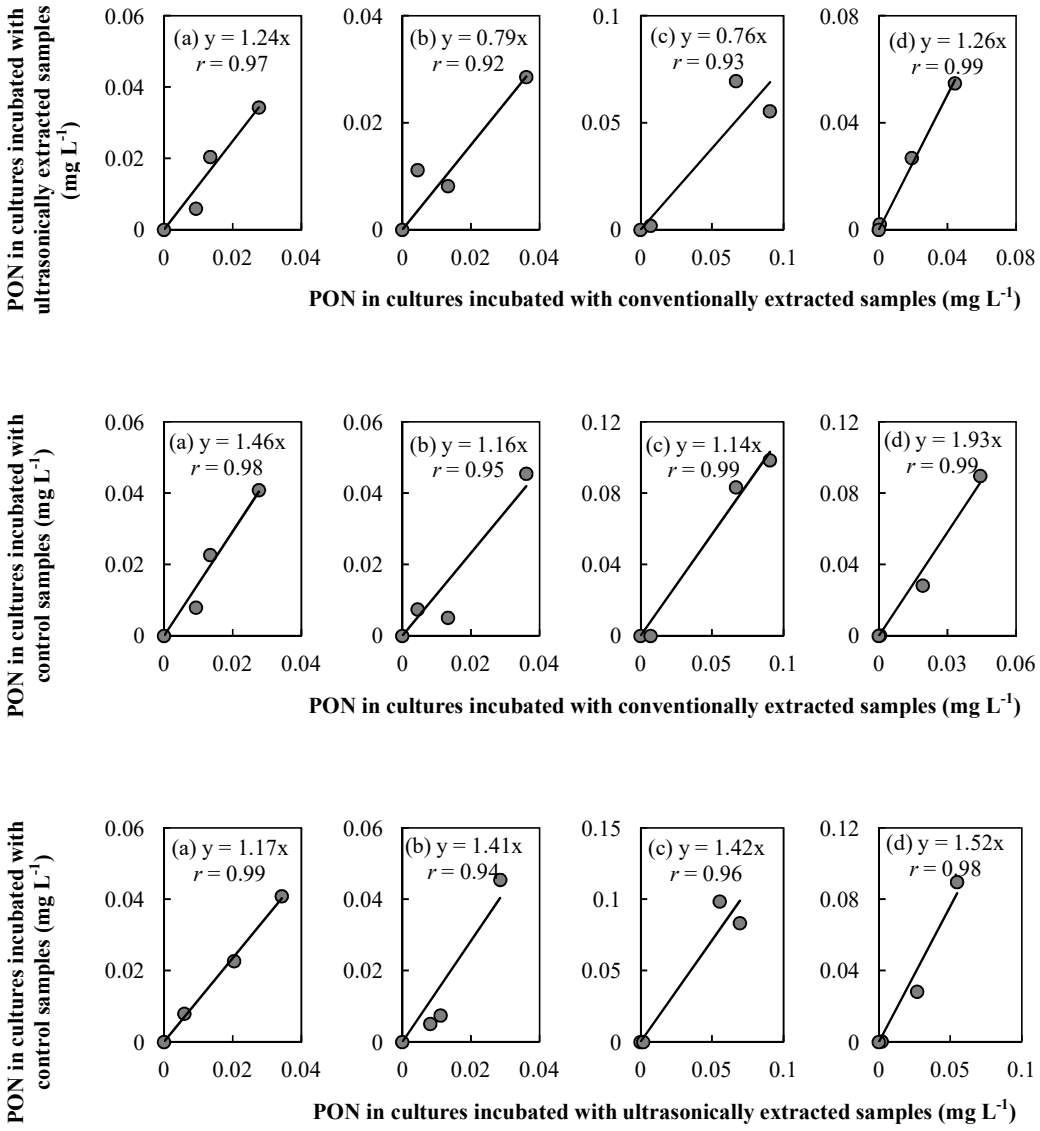

Figure 5-9. Similarity of PON concentrations in cultures incubated with conventionally extracted samples and with ultrasonically extracted samples. Comparison with the PON concentrations in cultures incubated with control samples. The designations a–d correspond to soil samples collected on 2011 January 26 and suspended sediment samples collected on 2014 September 5, September 11, and October 6, respectively.

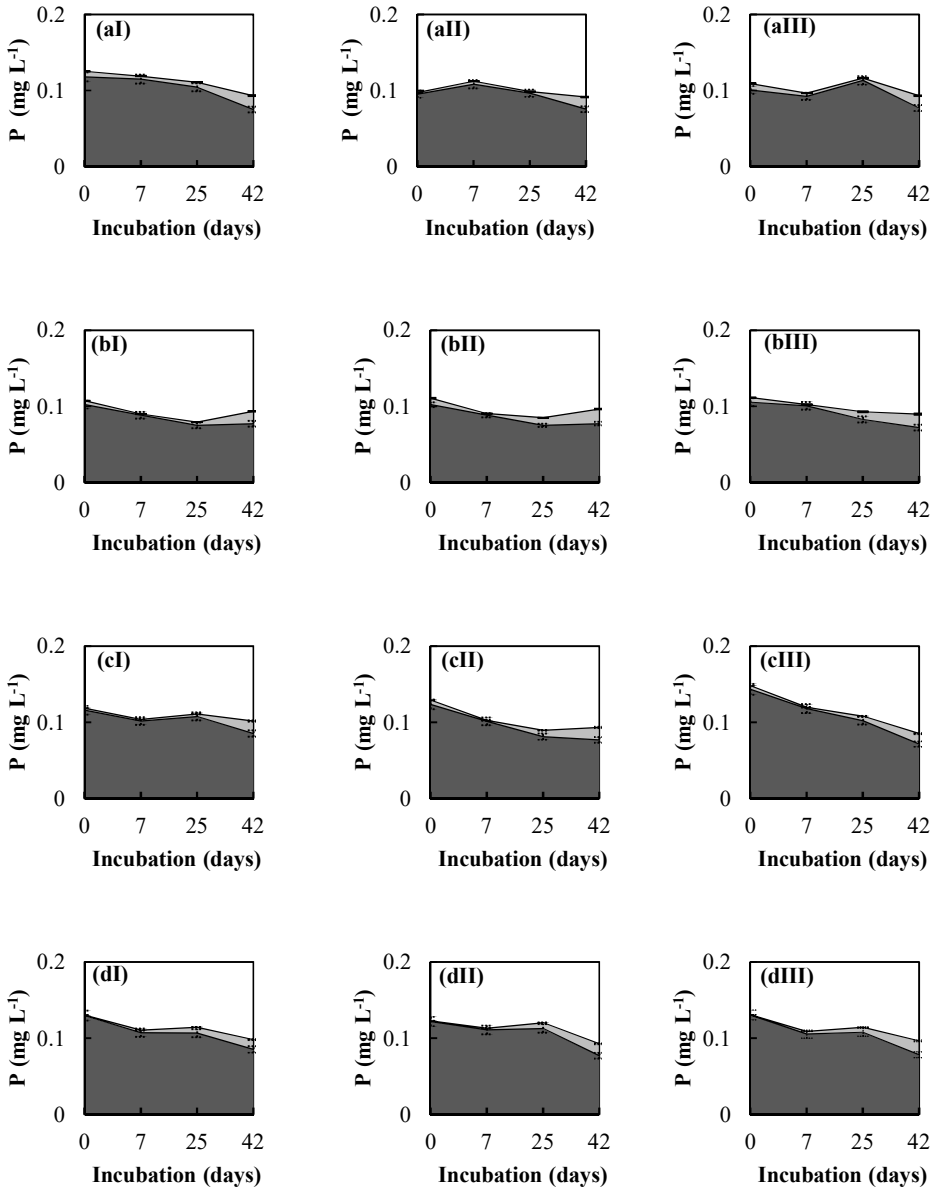

Figure 5-10. Concentrations of DP (■) and PP (■) in cultures of *M. aeruginosa* incubated with control samples (I), ultrasonically extracted samples (II), and mechanically extracted samples (III). The designations a–d correspond to soil samples collected on 2011 January 26 and suspended sediment samples collected on 2014 September 5, September 11, and October 6, respectively. Concentrations are means of triplicate values; bars indicate positive and negative errors.

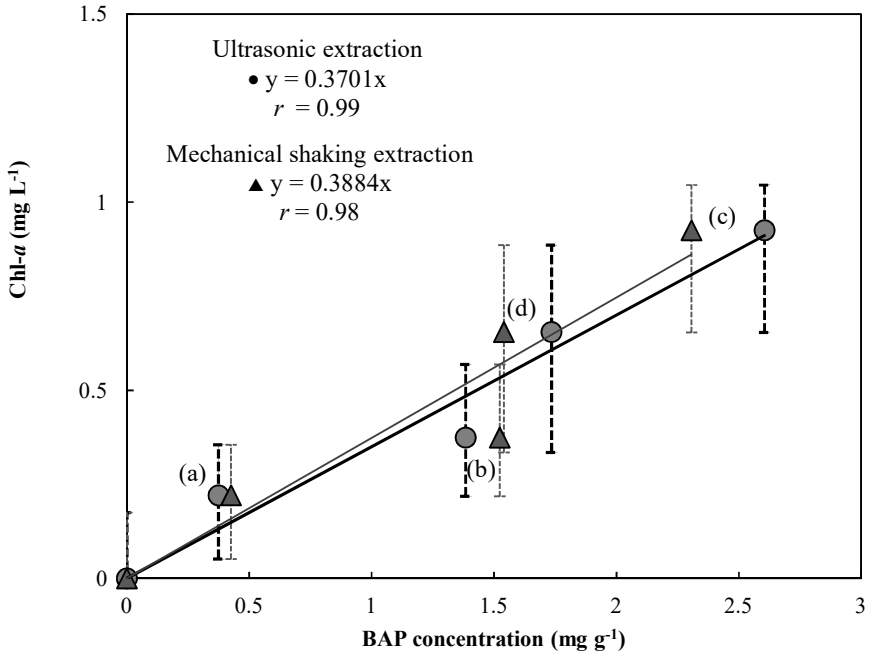

Figure 5-11. Relationship between Chl-*a* concentration at day 25 of incubation and BAP concentration obtained from extractions: (●) ultrasonic extraction and (▲) mechanical extraction. The designations a–d correspond to soil samples collected on 2011 January 26 and suspended sediment samples collected on 2014 September 5, September 11, and October 6, respectively. Concentrations are means of triplicate values; bars indicate positive and negative errors.

5.4.3 Bioavailability confirmation of extractable P

The potential bioavailability of extractable P was interpreted to be the growth of tested algae during incubation with the control sample (I) as the sole P source, that is, samples of dried soil or sediment prior to extraction. We assumed that the entire BAP fraction could be obtained by means of a single extraction, as has been reported previously

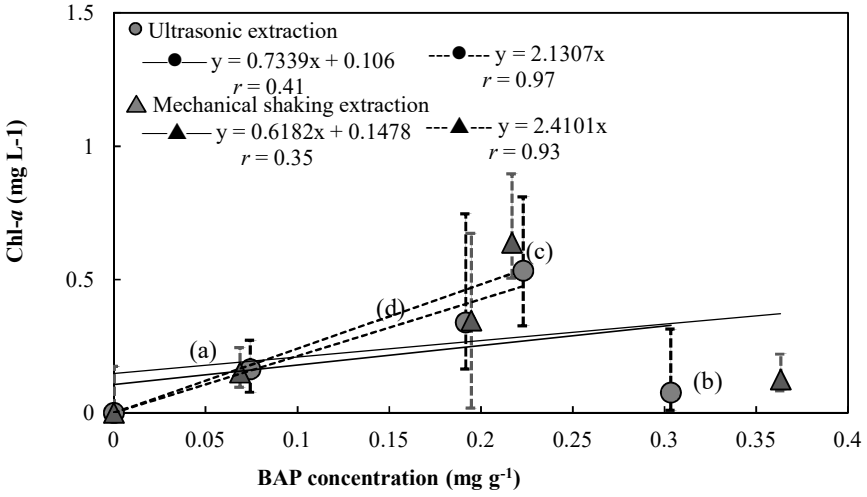

Figure 5-12. Relationship between Chl-*a* concentration at day 25 of incubation and P concentration extracted from the residues: (●) ultrasonic extraction and (▲) mechanical extraction. The designations a–d correspond to soil samples collected on 2011 January 26 and suspended sediment samples collected on 2014 September 5, September 11, and October 6, respectively. (—) Linear regression including data for sample b; (---) linear regression excluding data for sample b.

(Sharpley et al., 1991), that is, that the amount of extractable BAP was related to algal growth in the bioassays. We found that algal growth at day 25 of incubation, as indicated by Chl-*a* concentration, was strongly correlated (r = 0.99, $\alpha < 0.001$) with BAP mass fraction as quantified by ultrasonic extraction with 0.1 M NaOH (*Fig. 5-11*). This correlation was comparable (t = 0.08, p = 0.94 > 0.05) to that between *M. aeruginosa* production and BAP mass fraction as quantified by mechanical extraction (r= 0.98, $\alpha < 0.001$). Additionally, the correlation was similar to that previously reported (Sharpley et al., 1991) for the relationship between *Selenastrum capricornutum* growth and sediment BAP content determined by mechanical extraction in 0.1 M NaOH

(r = 0.96, α < 0.001, 29-day incubation). The strong correlation confirms the feasibility of using ultrasonic extraction for estimating particulate BAP and indicates that the amounts of P in each sample available for algal uptake were compatible with the amounts of extracted P. In other words, the correlation supports our initial hypotheses that a single ultrasonic extraction efficiently released all of the BAP from the soil and sediment samples.

5.4.4 Effects of nutrient deficiency on algal growth

The limiting nutrient in each medium was determined (*Table 5-1*), and the nutrient weight ratios were compared with the Redfield ratio (Dupas et al., 2015, Neill, 2005, Fagerbakke et al., 1996, Redfield et al., 1963) and the Healey and Hendzel ratios (Tsukada, 2006, Healey and Hendzel, 1979). Redfield (1963) reported that significant deviation of the C:N:P molar ratio from 106:16:1 indicates the supply of at least one nutrient is limited, whereas Healey and Hendzel (1979) suggested that a N:C molar ratio of < 0.12 and a P:C molar ratio of < 0.0077 (i.e. , a mass ratio of 0.0029) indicate N and P deficiency (in all cases, C, N, and P refer to particulate forms). In our study, starting from the first day of incubation, the C:N:P molar ratios in all the media greatly exceeded the Redfield ratio, which reinforces the aforementioned argument that P deficiency is more detrimental than the deficiency of other nutrients. It suggests that P was the algal–growth-limiting nutrient in all of the tested cultures. Additionally, the average N:C, N:P, and P:C mass ratios (*Table 5-1*) were clearly consistent with previously reported ratios (Fagerbakke et al., 1996, Tsukada, 2006) determined in studies of the relative amounts of C, N, and P in cultures of various bacteria collected from a variety of environments. In addition, the C:Chl-*a* and N:Chl-*a* ratios (*Table 5-3*)

were comparable to previously reported values (Rasul et al., 2014, Yacobi and Zohary, 2010), which proves that *M. aeruginosa* grew as well in our cultures with soil and suspended sediment samples as it did in the positive control cultures (*Fig. 5-3*). However, when we considered the bioavailable fractions in the total PP, we found that it was only in the cultures with the control samples (I) the P:C ratios were higher than Healey and Hendzel (1979) suggested as being indicative of P deficiency. It implies in the cultures with solid residues after mechanical and ultrasonic extractions, the algae grew well even though the supply of P was insufficient. This result suggests that in the absence of an external P source, *M. aeruginosa* could use cellular nutrients for their growth. This suggestion is consistent with our finding that Chl-*a* concentration increased slowly in the P-free negative control cultures, whereas the POC and PON concentrations were nearly constant or decreased slightly (*Fig. 5-3*). Moreover, the low P:Chl-*a* ratios (*Table 5-2*) also illustrate the substantial increase in Chl-*a* concentration, indicating algal growth, relative to the P concentration. The BAP fractions that probably remained in extraction residues II and III were measured by NaOH extraction of II with mechanical shaking (designated as "two-step mechanical extraction") or by NaOH extraction of III with ultrasonication (designated as "two-step ultrasonic extraction"). The weak correlation between BAP concentration determined by means of the two-step extraction and Chl-*a* concentration (*Fig. 5-12*) ($r = 0.41$, $\alpha < 0.5$) supports the notion that the extracted P was not truly bioavailable. The slopes for the single-step extractions (*Fig. 5-11*) were lower than the slopes for the two-step extractions (*Fig. 5-12*). This result demonstrates that the algae had more effort to grow in less extractable BAP in *Fig. 5-12* than in *Fig. 5-11*. The difference between the slopes in *Figs. 5-11* and *5-12* was not statistically significant (ultrasonic extraction: $t = 1.93$, $p = 0.10 > 0.05$; mechanical

extraction: $t = 1.26$, $p = 0.11 > 0.05$). We considered that in the extraction of solid residues, the use of NaOH in combination with ultrasonication might have suppressed the transformation of partially refractory P into SRP, which could be detected by our analyzer and which we assume to be BAP. However, in cultures with solid residues after mechanical and ultrasonic extractions, algal growth could be triggered not only by extractable P but also by cellular P. Therefore, we recommend a procedure involving a single-step extraction to determine BAP in the particulate phase.

Interestingly, when we omitted the data for the suspended sediment sample collected on 2014 September 5 (b), the correlation between extractable BAP and algal growth markedly improved (*Fig. 5-12*) but the slopes increased substantially and differed significantly from the data for the single-step extraction (*Fig. 5-11*) (ultrasonic extraction: $t = 8.86$, $p = 0.0003 < 0.05$; mechanical extraction: $t = 9.49$, $p = 0.0002 < 0.05$). This result implies that the relationship between algal growth and extractable BAP differed depending on whether we used a single-step extraction or a two-step extraction. The reason for the low P concentration in the suspended sediment sample (b) was unclear, but it might have been due to differences in the composition and characteristics of this sample, such as metal ions or re-adsorption of extracted P to solid residue surface, that led to a remarkable decline in the P concentration from the single-step extraction to the two-step extraction. The higher slopes in *Fig. 5-12* relative to *Fig. 5-11* emphasize that the algae we used for the bioassay adapted and grew well even though P was lacking in the culture environment. Further studies of the cellular mechanism of P uptake by algae, particularly *M. aeruginosa* are necessary to determine the factors that support algal growth in nutrient-deficient environments. Moreover, the growth of algae in P-free CB medium with no external P source (negative control), with

ultrasonically extracted samples (II), and with mechanically extracted samples (III) indicate that *M. aeruginosa* was inappropriate for AGP bioassays to evaluate the bioavailability in P-depleted media of extraction residues.

5.5. Summary

Algal growth in media containing ultrasonic extraction solid residues was similar level to that in media containing mechanical extraction solid residues (*Figs. 5-5, 5-7, 5-9*). This result confirms that ultrasonic extraction could provide similar results for quantification for BAP in soil and suspended sediment samples compared with the results for mechanical extraction. The high correlation between the amount of extracted BAP and algal growth at day 25 of incubation suggests that the BAP fraction could be obtained by means of a single-step ultrasonic extraction. Additionally, P was shown to be more important than C and N in limiting algal growth. However, the growth of *M. aeruginosa* in P-depleted media containing extraction solid residues and in media containing no external P source (negative control) implies that in the absence of an external source of P, the algae could utilize cellular nutrients for growth; that is, in addition to blooming as a result of the presence of excess nutrients in the environment, *M. aeruginosa* can grow even when the P concentration falls below a threshold level. Further consideration should be given to separation of P species in algal cells from particulate matter of soil or suspended sediment and to the cellular mechanisms of P uptake to clarify the growth-limiting factors under nutrient-depleted conditions. Finally, comparison of single-step extraction and two-step sequential extraction for BAP measurement suggests that the ultrasonic method involving a single-step extraction was effective for quantifying particulate BAP.

Table 5-2. Mass ratios of phosphorus, nitrogen, and carbon in *M. aeruginosa* cultures.

	N : C	N : P	P : C	C: N: P**	C: N: P***	BAP : C
Control soil (aI)	0.11 - 0.30	32.8 - 71.0	0.0020 - 0.0082	317:72:1	1284:157:1	0.0028
Soil after ultrasonic extraction (aII)	0.11 - 0.29	33.2 - 64.4	0.0027 - 0.0080	323:79:1	941:128:1	0.0005
Soil after conventional extraction (aIII)	0.12 - 0.31	27.7 - 57.8	0.0022 - 0.0082	314:73:1	1170:143:1	0.0006
Control sediment (bI)	0.11 - 0.29	31.9 - 75.6	0.0018 - 0.0080	324:81:1	1402:167:1	0.0031
Sediment after ultrasonic extraction (bII)	0.11 - 0.27	28.6 - 58.7	0.0022 - 0.0078	330:75:1	1197:147:1	0.0007
Sediment after conventional extraction (bIII)	0.11 - 0.28	34.1 - 66.2	0.0025 - 0.0082	316:73:1	1031:130:1	0.0006
Control sediment (cI)	0.14 - 0.29	35.5 - 128.6	0.0012 - 0.0080	321:78:1	2166:285:1	0.0044
Sediment after ultrasonic extraction (cIII)	0.11 - 0.28	37.1 - 96.4	0.0016 - 0.0073	355:83:1	1344:231:1	0.0003
Sediment after conventional extraction (cIII)	0.15 - 0.29	37.9 - 104.2	0.0015 - 0.0075	346:82:1	1604:189:1	0.0004
Control sediment (dI)	0.14 - 0.34	36.7 - 119.8	0.0013 - 0.0081	318:93:1	1952:265:1	0.0037
Sediment after ultrasonic extraction (dII)	0.10 - 0.34	36.9 - 84.9	0.0019 - 0.0075	343:93:1	1372:165:1	0.0004
Sediment after conventional extraction (dIII)	0.11 - 0.32	36.4 - 74.5	0.0015 - 0.0078	333:97:1	1698:188:1	0.0004
Negative control	0.22 - 0.28	104.6 - 163.5	0.0013 - 0.0022	500:92:1	1364:259:1	0.0013
Positive control 0.1 mg L^{-1}	0.12 - 0.28	36.0 - 67.9	0.0018 - 0.0074	487:80:1	1421:150:1	0.0053
Positive control 0.2 mg L^{-1}	0.19 - 0.30	17.3 - 47.1	0.0041 - 0.0176	269:49:1	633:104:1	0.0096
References	Fagerbakke et al.(1996): 0.17 - 0.29* i.e. 0.15 - 0.25	Tsukada et al. (2006): 15.0 - 98.0* i.e. 33.2 – 217.0	Tsukada et al. (2006): 0.00098 - 0.017* i.e. 0.0003 - 0.0066	• Redfield ratio (1963): 106C:16N:1P* • Healey & Hendzel (1979): N deficiency in algae when N:C < 0.12* P deficiency in algae when P:C < 0.0077* (i.e. mass P:C < 0.0029)		

* the atomic ratio; ** the average atomic ratio at the initial day (0 day); *** the average atomic ratio at the 25th day.

Table 5-3. Mass ratios of phosphorus, nitrogen, and carbon relative to Chl-*a* in *M. aeruginosa* cultures at day 25 of incubation.

	C : Chl-*a*	N : Chl-*a*	P : Chl-*a*
Control soil (aI)	159.0	22.7	0.3
Soil after ultrasonic extraction (aII)	150.0	23.8	0.4
Soil after conventional extraction (aIII)	177.4	25.2	0.4
Control sediment (bI)	162.5	22.6	0.3
Sediment after ultrasonic extraction (bII)	213.2	30.5	0.5
Sediment after conventional extraction (bIII)	237.9	34.9	0.6
Control sediment (cI)	93.5	14.3	0.1
Sediment after ultrasonic extraction (cII)	71.3	14.3	0.1
Sediment after conventional extraction (cIII)	99.4	13.7	0.2
Control sediment (dI)	101.2	16.0	0.1
Sediment after ultrasonic extraction (dII)	121.3	17.0	0.2
Sediment after conventional extraction (dIII)	152.5	19.7	0.2
Negative control	147.5	32.7	0.3
Positive control 0.1 mg L^{-1}	73.0	19.4	0.2
Positive control 0.2 mg L^{-1}	49.4	11.9	0.3
References	Yacobi & Zohary (2010): 39.0 - 182.0, Rasul et al. (2014): 94.2[26],	Rasul et al. (2014): 16.6,	Rasul et al. (2014): 2.3, Chen et al. (2011): 0.2 - 1.2

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Chapter 6 Conclusion

Bioavailable phosphorus (BAP) showed more closely related to the growth of algae in water environment, thereby it demonstrates to be a better indicator of eutrophication than total phosphorus (TP) which have been using in water quality standards. BAP in particulate forms has been more attracted because of P's strong affinity for particles of soil and sediment. Agricultural activity can be a harmful P pollution source due to its usage of intensive P-containing fertilizers. When a rainfall or flood occurs, drains or overland routes wash out these excessive P soils into aquatic environment, accumulating in suspended sediments. This dissertation investigated the particulate BAP fraction from one soil sample and three river suspended sediment samples from a representative agricultural source in the watershed of Umeda River, a river flow to Mikawa Bay which is one of the most eutrophic regions of Japan's main island. The proportion of BAP in total PP was consistent to previous studies. It reinforced the potential P pollution from paddy fields in Umeda River following to wide-spreading eutrophication in Mikawa Bay. Henceforth BAP in particulate forms should be considered for a better management of eutrophication in watersheds.

The conventional methods for quantifying particulate BAP are too time-consuming thus limit the amount of analyzed sample. A conventional single extraction using mechanical shaking requires 17 h only for extraction and up to several weeks for the whole procedure. In order to shorten the working process, an application of ultrasonic treatment on the extraction of BAP in particulate forms especially soil and suspended sediment samples from agricultural sources was assessed in this study. The most optimal conditions of ultrasonic extraction were identified. The proposed ultrasonic

method allowed an extraction time of only 1 min whereas the mechanically shaking method required 17 hours for BAP extraction. Based on this promising result, we will be able to develop a new standard method for quantifying particulate BAP that can be more suitable for routine analyses in eutrophication monitoring.

We also evaluated the bioavailability of the extractable BAP by AGP bioassays using P-starved *M.aeruginosa*. The growth of algae in the media containing samples after the ultrasonic extraction was illustrated at identically similar level with those in the media containing samples after the conventional extraction. It strengthened that ultrasonic treatment could provide a similar quantification BAP in soil and suspended sediment samples when using the conventional extraction. The high correlation between the amount of extracted BAP and the algal growth at 25-day incubation suggested that the BAP fraction could be entirely obtained in once extraction. Additionally, P was confirmed more important than other nutrients (C and N) in limiting algal growth. The concept underlying the bioassay is that P is a critical requirement for optimum growth of tested algae in a P-depleted culture medium, ie. *M.aeruginosa* would be difficult to grow in media containing solid residues after extraction. However, our result appeared to contradict this hypothesis. The growth of *M.aeruginosa* in the media containing samples after extraction and negative control implied the algae could utilize their cellular nutrients to develop without external P. In this case, *M.aeruginosa* might be unsuitable for evaluate P depletion in AGP bioassays. Therefore, various blue-green algae should be examined to get fate of bioavailable phosphorus in environment. Moreover, further consideration should be given to the intracellular mechanisms of cyanobacteria to clarify the growing possibly limiting factor under nutrient depletion.

Finally, these findings suggested that the proposed method with single-step ultrasonic

extraction provided a promising alternative for quantifying BAP in particulate forms that can shorten the working process, thereby more samples can be analyzed.