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博士論文

Application of stoichiometric approaches for identification of relationships between
resources and benthic invertebrates in stream ecosystems

(河川生態系における資源-底生無脊椎動物関係をひもとく
生態系化学量論的アプローチ)

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Chapter 1

General Introduction

Relationships between resources and consumers have been central focus of ecology, and are keys to understand the structures of ecosystems (Schmid-Araya & Schmid, 2002; Ritchie 2010; Schmitz 2010). The stoichiometric proportion among resources and consumers becomes very informative variable to understand the complex systems (Sterner & Elser 2002). Therefore, numerous studies that focused on the stoichiometry of the resources and consumers conducted in the forest (e.g. Elser et al. 2000a; 2000b), glassland (e.g. Mulder & Elser 2009; González et al. 2010), soil (e.g. Peñuelas & Sardans 2009; Marichal et al. 2011), marine (e.g. Elser et al. 1994; Zimmerman et al. in press), lake (e.g. Urabe et al. 2002; Frost et al. 2002) and stream (e.g. Cross et al. 2003; Hladysz et al. 2009) ecosystems. Especially, stoichiometric imbalances in carbon : nutrient ratios among resources and consumers have been paid attentions over the years. Basal resources in food webs vary widely in their elemental composition and its quality (Cross et al. 2005), whereas consumers often operate within more tightly-constrained limits (Sterner & Elser 2002). Consequently, imbalances can occur when the elemental composition of the food resource is not suitable for the elemental requirements of the consumer (Pandian & Marian 1986; Sterner & Hessen 1994; Sterner 1997).

Many previous studies estimated the stoichiometric imbalances among resources and consumers, and demonstrated the effects on growth, reproduction and community composition (Sardans et al. 2012; Hessen et al. 2013) (Allow A in Fig. 1). And then factors those alter stoichiometry of resources were focused and demonstrated the effects on consumers (e.g. Urabe et al. 2002; Cross et al. 2007; Davis et al. 2010) (Allow B in Fig. 1). However, some studies showed the stoichiometry of consumers'

body differ among species and the surrounding environments (e.g. Evans-White et al. 2005; Frost et al. 2010; Persson et al. 2010; Small et al. 2010). Although we have to estimate whether there are the ecological implications of stoichiometric differences among consumers, there are very few studies that verified the problem.

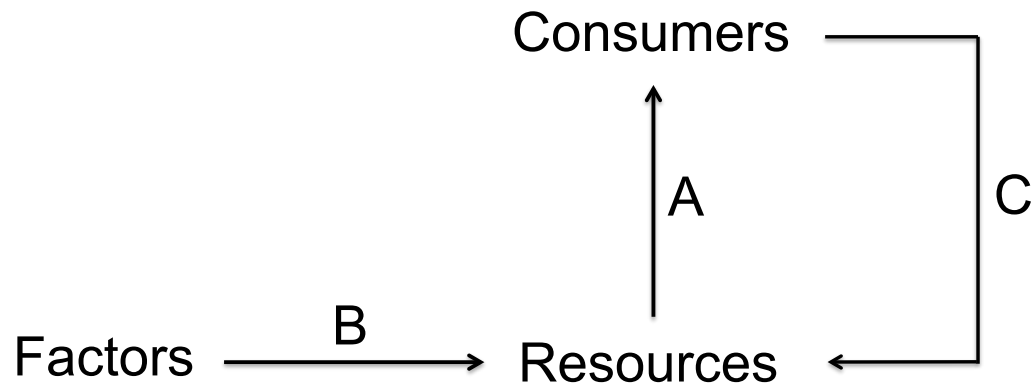
In stream ecosystems, many studies showed the imbalances has repercussions for invertebrate growth, reproduction and C assimilation efficiencies, which in turn influence ecosystem functions, such as litter decomposition and material flow rates between trophic levels (Allan & Castillo 2007; Cross et al. 2007; Woodward 2009). However, there are still many points of view and approaches to identify the relationships between resources and benthic invertebrates in the stream ecosystem using stoichiometric theory. Moreover, we have to consider relationships between stoichiometry of resources and another environmental factors, and assess precisely the significance of stoichiometry of resources for consumers.

Objectives of this thesis

In this study, I addressed the relationships between resources and benthic invertebrates in stream ecosystem from various viewpoints using stoichiometric theory. In Chapter 2 to 4, I focused on the factor that alters stoichiometry of resources (Allow B in Fig. 1-1). In Chapter 2, I demonstrated that light intensity affected growth and reproduction of a snail grazer in an oligotrophic stream ecosystem through changes in the stoichiometric proportion and biomass of periphyton (Ohta et al. 2011). This is the first study that demonstrated effects of light intensity on the relationship between

periphyton and a grazer using stoichiometric theory. In Chapter 3, I illustrated effects of subsidiary calcium on invertebrate communities in some streams, and implied that subsidy is an important view points to estimate stoichiometric relationships between resources and consumers in stream. Then, I demonstrated the amount of subsidiary calcium is varied by catchment vegetation, and affected the abundance and survival of crustaceans in streams (Ohta et al. in press). This is the first study showing the importance of the terrestrial vegetation to stream invertebrate through supply of subsidiary calcium. In Chapter 4, I estimated simultaneously the effects of stoichiometry of resources and another environmental factor on ecosystem function and community of stream invertebrates. I estimated whether effects of nutrient enrichment on litter decomposition and invertebrate community were altered by the light availability. Then, I demonstrated synergic effects of light and nutrient availability on litter decomposition and invertebrate community in a stream. This is the first study to show that the effects of nutrient enrichment can be altered by light availability. In Chapter 5, I focused on the ecological implications of stoichiometric differences among consumers (Allow C in Fig. 1-1). I estimated whether litter decomposition was affected by diversity of the detritivores in a stream ecosystem using stoichiometric theory. Then, I showed stoichiometric differences among detritivores played an important role in the relationships. Therefore, I found a breakthrough in the important question of ecology (i.e. relationships between biodiversity and ecosystem function) by applying stoichiometric theory. Finally, in Chapter 6, I reviewed the previous studies and discussed the importance of relationships between resources and stream invertebrates to

describe stream ecosystem.



A: Effects of the resources stoichiometry on its consumers

B: Factors those alter stoichiometry of resources

C: Effects of stoichiometric differences among consumers on ecosystem function

Fig. 1-1 The conceptual diagram of study that identifies relationships resources and consumers using stoichiometric theory.

Chapter 2

**Light intensity regulates growth and reproduction of a snail grazer through
changes in the quality and biomass of stream periphyton**

INTRODUCTION

The ratio of carbon : nitrogen : phosphorus (C : N : P) of producer tissues is a very important factor regulating the growth and reproduction of primary consumers, while the ratios of N and P to C are often much lower in producers than in the herbivores that consume them (Elser & Hassett 1994; Sterner & Elser 2002; Hillebrand et al. 2004; Liess & Hillebrand 2005). These stoichiometric imbalances between producers and consumers are likely to impose constraints on the growth and reproduction of consumers (Elser et al. 2000a; Plath & Boersma 2001; Frost et al. 2002). Therefore, determination of the forces driving C : N and C : P ratios may reveal the mechanisms underlying ecosystem processes such as matter flow between trophic levels and aspects of consumer population dynamics including reproduction (Olsen et al. 1986; Sterner & Elser 2002; Frost et al. 2005).

Producers that serve as food resources for primary consumers acquire carbon by photosynthesis and take up nitrogen and phosphorus from the surrounding environment. Thus, C : N and C : P ratios in producers are governed by light intensity and nutrient availability in both freshwater (Sterner et al. 1997; Elser et al. 2000a; Sterner & Elser 2002) and terrestrial ecosystems (Nakamura et al. 2008; Takafumi et al. 2010). The C : N and C : P ratios of producers are in theory expected to be higher under high-light conditions than under low-light conditions at given nutrient concentrations because more carbon is assimilated under high-light conditions. This pattern has been observed in various ecosystems, including streams (Fanta et al. 2010), lakes (Urabe & Sterner 1996) and terrestrial ecosystems (Elliott & White 1994). This means that

herbivore growth rates are more likely to be limited by food quality (e.g. C : N and C : P ratios) in environments with high light-to-nutrient ratios (Sternner et al. 1997). Conversely, herbivore growth rates are more likely to be limited by food quantity under low-light conditions where photosynthetic activity and producer biomass are low (Urabe & Sternner 1996). Thus, in oligotrophic environments, herbivore growth rates might be limited under both high-light conditions and low-light conditions. The light : nutrient hypothesis (LNH) therefore predicts that herbivore growth rates should be maximal at intermediate light intensity in oligotrophic environments (Sternner et al. 1997).

In the previous studies, the LNH has primarily been tested by studying the interactions between phytoplankton and zooplankton in lake ecosystems, and these studies have supported the LNH prediction that zooplankton growth rates are limited by food quantity under low light intensities and by food quality under high light intensities in oligotrophic environments (Hessen et al. 2002; Urabe et al. 2002; Hall et al. 2007). A number of studies have examined the interactions between periphyton and herbivores in streams (Rosemond et al. 1993; Rosemond et al. 2000; Stelzer & Lamberti 2001) and lakes (Liess & Hillebrand 2005; Fink & Von Elert 2006) and have found that C : N and C : P ratios in periphyton are mainly affected by nutrient concentrations in the water column and grazer growth is restricted by the availability of foods with high C : N and/or C : P ratios. However, none of these studies demonstrated the interactions between periphyton and grazers predicted by the LNH (Hill et al. 2010). In this study, I tested the LNH in a stream ecosystem where light conditions were horizontally

heterogeneous. In addition, growth rates of slow-growing herbivores are less affected by C : N and C : P ratios, even when these ratios are high (Sternner & Elser 2002; Frost et al. 2006). Therefore, I used fast growing juvenile snails in the present study.

Foods with low nutritional value affect not only the growth of herbivores but also their reproduction (Smith 1979; Urabe & Sternner 2001; Færøvig & Hessen 2003; Tibbets et al. 2010). Many studies that have examined the effects of food resources on reproduction focused on carbon-allocation strategies in invertebrate reproduction in freshwater ecosystems, such as the number of eggs or egg size (Tessier et al. 1983; Tessier & Consolatti 1991; Lampert 1993; Boersma 1995). However, some herbivores invest phosphorus in gonad tissues during the breeding season (Ventura & Catalan 2005), and their hatching rate decreases when the rate of phosphorus allocation to the gonad tissue declines, even if the number of eggs or egg size are not affected (Urabe & Sternner 2001). No freshwater study has yet examined the effect of light conditions on herbivore reproduction through stoichiometric changes, despite the possibility that light conditions can indirectly influence reproduction in oligotrophic environments by changing the C:N and C:P ratios of producers. I predicted that light conditions would have an indirect effect on herbivore reproduction by altering the C : N and C : P ratios of producers.

I experimentally tested under oligotrophic conditions the effects of light conditions, via stoichiometry, on the growth rates and reproduction of snails held at a density comparable to field conditions. I tested the following predictions: (1) periphyton biomass would increase, and C : N and C : P ratios in periphyton would decrease with

enhanced light intensity, (2) snail growth rate would be highest at an intermediate light intensity and (3) the phosphorus content of gonadal tissue would be highest at an intermediate light intensity.

METHODS

Study snail and field sampling

The herbivorous snail *Gyraulus chinensis* Dunker was collected from Horonai Stream, which runs through the Tomakomai Experimental Forest of Hokkaido University, southwestern Hokkaido, Japan (TOEF: 42°43'N, 141°36'E). This stream originates from a spring and its bed is underlain by pumice, and have very low nutrient levels are present in the stream water throughout the year [inorganic nitrogen: 20 $\mu\text{g L}^{-1}$ (± 5.1 SE), total phosphorus: 1.0 $\mu\text{g L}^{-1}$ (± 0.26 SE)]. *G. chinensis* is a dominant species in the middle reaches of the stream. Congeneric species are widely distributed in streams and freshwater ponds in Asia, America and Europe (Dussart 1979; Parashar & Rao 1988; Habe 1990). The snails have an adult shell diameter of c. 5 mm (Habe 1990), a lifespan of about 6 months, and they breed in spring and autumn (Dussart 1979). I collected snails from within a 5 · 10 m plot on the streambed, in which current velocity and water depth were relatively uniform (current velocity: $10 \pm 2 \text{ m s}^{-1}$, water depth: $20 \pm 5 \text{ cm}$). The snails were kept for 21 days prior to the experiment in a reserve experimental channel similar to those used in the experiment. The bottom of the channel was covered with unglazed ceramic tiles that had been placed previously in a large basket constructed of 1 mm mesh netting in the streambed plot for 21 days to allow periphyton

colonisation. During the snail acclimatisation period, light was maintained at $50 \text{ } \mu\text{mol s}^{-1} \text{ m}^{-2}$ during the daytime (6:00–18:00). The juvenile snails used here (3.0–3.2 mm) have higher specific growth rates than adult snails because they invest most of their energy into growth instead of reproduction (Brendelberger 1995). *G. chinensis* has a short life span and the snails approached maturity at the end of the experiment when I could estimate both growth and reproduction. The initial diameters of individuals were measured using a digital vernier calliper with a precision of 0.01 mm (CD-20B; Mitutoyo, Kawasaki, Japan). The initial dry mass of *G. chinensis* individuals was calculated using an allometric equation:

$$\log_{10}(\text{soft body}) = 2.514 \times \log_{10}(\text{shell diameter}) - 1.630$$

$$(n = 250, R^2 = 0.964; P < 0.001)$$

Final dry mass was measured directly.

Experimental system

The experiment was conducted from 19 July to 18 September 2010. I manipulated light intensity in eight semi-cylindrical channels (size: $200 \times 35 \times 20 \text{ cm}$, see Kuhara et al. 1999; Nakano & Miyasaka 2001) built to simulate stream environments with physical conditions held constant (current velocity, water depth and water temperature). Water was supplied at a constant rate to all channels from a well dug into the stream bank, and the channels were aerated using aquarium pumps. The nutrient concentrations in the

well water were similar to those in the natural stream water (Table 2-1). Sixteen treatment areas were created by bisecting each channel with a shade curtain (Fig. 2-1). Four different light conditions (50, 300, 1000 and 1500 $\mu\text{mol s}^{-1} \text{m}^{-2}$) were created across the 16 treatment areas by controlling the distance from each channel to a light-emitting diode (LED) light (36 collimated LED dual-colour grow-light panels 2510R + B; LED wholesalers, Burlingame, CA, U.S.A.; Fig. 2-1). The LED only released photosynthetically active wavelengths. The darkest condition (50 $\mu\text{mol s}^{-1} \text{m}^{-2}$) was very similar to the light conditions in areas of the Horonai Stream beneath dense canopy (Nakano & Murakami, 2001), and the brightest condition (1500 $\mu\text{mol s}^{-1} \text{m}^{-2}$) corresponded to exposure to direct sunlight. The other conditions were created to identify the point at which light had a negative affect on snail growth and reproduction. Light (18:00–6:00) and dark periods (6:00–18:00) were alternated using a timer attached to an electrical supply. Light interference among the treatments was avoided by using shade curtains. Thirty-two nylon cages (11 × 11 × 20 cm, 2.5-mm mesh) were placed in each light condition, and a tile (10 × 10 cm) colonised by periphyton (for 21 days on the natural stream bed) was placed in each cage. Twenty-four of these cages had one *G. chinensis* individual, and the remaining cages had no snails. The mean density of *G. chinensis* in the stream plot was about one snail per 9 × 9 cm, corresponding to the density observed in the field. The cages were randomly distributed among the light conditions (Fig. 2-1). which were maintained for 60 days. During the experimental period, cages were cleaned with a brush and a small net every 4 days to remove floating algae and algae attached to the sides.

Treatment of samples

Water temperature was monitored every 30 min during the experimental period using a temperature sensor with a logger (Tidbit v2 UTBI-001; Onset, Bourne, MA, U.S.A.) set at the downstream end of each channel. Current velocity in each channel was measured every 2 days using a current meter (VR-201; KENEK, Tokyo, Japan). Water samples were collected from each channel every 4 days, filtered through GF/C filters (Whatman No. 1822, U.K.) and then frozen at - 80 °C for chemical analyses. These water samples were analysed for dissolved inorganic nitrogen using standard methods (APHA 2005) and total phosphorus using an ICP Atomic Emission Spectrometer (ICPE-9000; Shimadzu, Kyoto, Japan).

Periphyton samples were collected on the final day of the experiment by brushing the surface of the tiles and rinsing with distilled water. These suspensions were filtered onto glass filters (Whatman No. 1822, Maidstone, U.K.) within 24 h of sampling. Suspensions from cages where snails were present were divided into two subsamples and filtered separately. Filtered periphyton samples were dried at 60 °C for 24 h in a drying oven (NDO-450ND; Eyela, Tokyo, Japan) and stored in a deep freezer at -80 °C until

chemical analysis. Filtered samples from cages without snails were ashed at 490 °C for 2 h in an electric muffle furnace (KM-420; Advantec, Japan). Ash-free dry mass (AFDM) was calculated as the difference in mass before and after ashing to estimate periphyton biomass. Periphyton subsamples from cages with snails were ashed at

490 °C for 2 h, weighed and extracted with 15 mL 1 M HCl at 80 °C for 1 h. Then the extracts were analysed for phosphorus concentration per AFDM using the ICP atomic emission spectrometer. The remaining subsamples were analysed for carbon and nitrogen concentrations per AFDM using a C : N analyzer (NC-900; Sumitomo, Osaka, Japan). The C : N and C : P ratios of periphyton samples from cages with snails were calculated from these results, and the periphyton biomass in these cages was calculated as the sum of AFDM between the two subsamples.

Gyraulus chinensis samples were collected on the final day of the experiment. Shell diameter was measured for each individual, and shell growth was calculated as the difference in shell diameter before and after the experiment. Then, within 24 h of sampling, the snails were removed from their shells and their bodies were separated into gonad and muscle tissues under a dissecting microscope. These tissues were dried at 60 °C for 24 h in a drying oven and dry mass was measured for both types of tissue. Total body mass was calculated as the sum of the masses of the two tissues, and the percentage of gonad tissue by mass was calculated. These dried tissues were stored in a deep freezer at -80 °C for chemical analysis. AFDM and the absolute quantities of phosphorus in gonad and muscle tissues were calculated using the same methods as for periphyton; P concentrations in gonad tissues and P allocations to gonad tissues per AFDM were calculated. Snails that died during the experiment (three individuals in each light condition of 50, 1000 and 1500 $\mu\text{mol s}^{-1} \text{m}^{-2}$, two individuals at 300 $\mu\text{mol s}^{-1} \text{m}^{-2}$) were removed from the channels and were not included in the analyses.

Statistical analyses

Periphyton biomass was analysed using a two-way analysis of variance (ANOVA) with light intensity and snail presence as independent variables, followed by a post-hoc comparison using the Tukey–Kramer test. The C : N and C : P ratios of periphyton from cages with snails, growth in shell diameter, P content and concentration in gonad tissues, P allocation rate to gonad tissue (arcsine transformed), and gonad mass were analysed using one-way ANOVAs with light intensity as an independent variable, followed by post- hoc comparisons using the Tukey–Kramer test. The coefficient of each explanatory variable (i.e. periphyton biomass, C : N ratio and C : P ratio) for the dependent variable of growth in shell diameter was estimated using a generalised linear model (GLM). I used the likelihood ratio test to determine whether the data supported selected models over a null model. Data were analysed separately by low ($50 \mu\text{mol s}^{-1} \text{m}^{-2}$) and high light conditions (300, 1000 and $1500 \mu\text{mol s}^{-1} \text{m}^{-2}$) because we predicted that the main determinants of snail growth under the low and high light conditions would be periphyton quantity and C : P ratios in periphyton, respectively. I selected best-fit models in a stepwise fashion using Akaike’s information criterion and used simple linear regression analysis to examine the contribution of each significant explanatory variable of growth in shell diameter and body growth rate. All statistical analyses were performed using the software R version 2.9.2 (R Development Core Team 2008).

RESULTS

Periphyton

High-light conditions led to low food quality but high food quantity, whereas low-light conditions led to high food quality but low food quantity. Periphyton biomass differed significantly between light intensities (two-way ANOVA : $F_{1,30} = 191.93$, $P < 0.001$). Light intensity had a significant positive effect on periphyton biomass (Fig. 2-2, Tukey–Kramer tests, $P < 0.05$). These results suggest that light controlled periphyton biomass in this system. In addition, snail presence had a significant negative effect on periphyton biomass, but only at the lowest light intensity (Fig. 2-2, Tukey–Kramer tests, $P < 0.001$). The C : N (one-way ANOVA: $F_{3,82} = 35.26$, $P < 0.001$) and C : P ratios (one-way ANOVA: $F_{3,82} = 7.11$, $P < 0.001$) in periphyton differed significantly between the low (50 and 300 $\mu\text{mol s}^{-1} \text{m}^{-2}$) and high light conditions (1000 and 1500 $\mu\text{mol s}^{-1} \text{m}^{-2}$; Fig. 2-3, Tukey–Kramer tests, $P < 0.001$).

Snail growth

Growth in shell diameter differed significantly among light intensities (one-way ANOVA: $F_{3,82} = 7.58$, $P < 0.001$), being significantly higher at 300 $\mu\text{mol s}^{-1} \text{m}^{-2}$ than under other light conditions (Fig. 2-4; Tukey–Kramer tests, $P < 0.05$), and body growth rate showed similar results (one-way ANOVA: $F_{3,82} = 7.85$, $P < 0.001$, Tukey–Kramer tests, $P < 0.05$). The mean percentage of gonad tissue mass relative to total body mass was c. 29.6% (± 1.9 SE) and did not differ significantly under different light conditions (one-way ANOVA: $F_{3,82} = 2.02$, $P = 0.18$).

For growth in shell diameter, stepwise GLM analyses identified periphyton biomass as the best-fit explanatory variable under low-light condition (50 $\mu\text{mol s}^{-1} \text{m}^{-2}$)

and periphyton C : P ratio as the best-fit model under high-light conditions (300, 1000 and 1500 $\mu\text{mol s}^{-1} \text{m}^{-2}$; Table 2-2). The results for body growth rate were similar to those for growth in shell diameter (Table 2-2). Regression analysis indicated that growth in shell diameter was positively correlated with periphyton biomass in the low-light condition (Fig. 2-5), and the results for the correlation between body growth rate and periphyton biomass were similar ($R^2 = 0.162$, $P = 0.009$). Shell growth was negatively correlated with periphyton C : P ratios in high-light conditions (Fig. 2-5). The coefficient between body growth rate and periphyton C : P ratio was similar to that between shell growth and periphyton C : P ratio, but the significance level was marginal ($R^2 = 0.127$, $P = 0.058$). These relationships between shell growth and periphyton parameters were similar when data for light conditions of 50 and 300 $\mu\text{mol s}^{-1} \text{m}^{-2}$ (periphyton biomass versus shell growth: $R^2 = 0.171$, $P = 0.029$) were separated from those for light conditions of 1000 and 1500 $\mu\text{mol s}^{-1} \text{m}^{-2}$ (periphyton C : P ratio versus shell growth: $R^2 = 0.132$, $P = 0.014$).

Phosphorus content and allocation in snails

The absolute quantity of phosphorus in gonad tissue and the body differed significantly among light intensities (one-way ANOVA : $F_{3,82} = 21.27$, $P < 0.001$ for the gonad, $F_{3,82} = 51.00$, $P < 0.001$ for the body): that in the gonad tissue at 300 $\mu\text{mol s}^{-1} \text{m}^{-2}$ was significantly higher than that under the other light conditions. The concentration of phosphorus in gonad tissue also increased with increasing light intensity (Fig. 2-6, Tukey–Kramer tests). Because the percentage of gonad tissue mass relative to total

body mass was almost constant, rates of P allocation to gonad tissue (arcsine transformed) increased with increasing light intensity and were significantly different between the 50 and 1500 $\mu\text{mol s}^{-1} \text{m}^{-2}$ light conditions (Fig. 2-6, $P = 0.045$).

DISCUSSION

This is the first study to provide evidence in support of the LNH between periphyton and herbivores. Periphyton biomass increased with higher levels of light intensity, and C : N and C : P ratios in periphyton were elevated under high-light conditions (supporting prediction 1). The growth rate of the herbivore and the phosphorus content of its gonad tissue were maximised at an intermediate light intensity under oligotrophic conditions (supporting predictions 2 and 3).

Response of periphyton to light conditions

Periphyton biomass increased with light intensity. However, the availability of light and nutrients affects not only producer productivity but also their nutrient content (Sternner et al. 1997; Fanta et al. 2010). Higher C-fixation rates lead to more C in producers, but light can also increase nutrient competition as well as C : N and C : P ratios in producers (Sternner et al. 1997). Moreover, light can reduce periphyton nutrient content, but only in oligotrophic environments (Fanta et al., 2010). Because the water in our experimental channels had very lower nutrient concentrations (Table 2-1), the periphyton C : N and C : P ratios were higher in the high-light condition. However, Hill & Fanta (2008) did not find a negative correlation between light and periphyton P

content in oligotrophic laboratory streams. The main reason for their result is that the light intensity they used was too low ($< 80 \mu\text{mol s}^{-1} \text{ m}^{-2}$; Fanta et al. 2010). Our experimental system included very high light intensities, which produced the negative correlation. Therefore, our results showed that periphyton were produced in low quantity and high quality under low-light conditions, and in high quantity and low quality (i.e. high C : N and C : P ratios) under high-light conditions.

Effects of light conditions on snail growth

C : P ratios in periphyton were selected as the best-fit explanatory variable for snail growth rate under high- light conditions and showed an overall negative effect. This finding may indicate that the growth rates of *G. chinensis* under high-light conditions were limited by P deficiency. Herbivores in freshwater ecosystems maintain low, constant C : P ratios in their bodies relative to producers by strict homeostasis (Hessen 1990; Liess & Hillebrand 2005; Fink et al. 2006). However, the inflection point that is predicted by the LNH can vary among herbivores because of essential differences in body nutrient content and their homeostatic strictness (Sternner et al. 1997; Sternner & Elser 2002). The C : N : P ratio varies considerably among benthic macroinvertebrate taxa, while the C : N : P ratios of molluscs and aquatic insects also vary among genera, and benthic grazers are less homeostatic than zooplankton (Evans-white et al. 2005). However, nutrient concentrations in snails are generally not low, and nutrient demand varies among seasons (Persson et al. 2010). Conceivably, demand for nutrients is elevated during the growing and reproductive seasons. Our experimental period

encompassed both seasons for *G. chinensis*. Frost et al. (2006) estimated threshold element ratios (TER) at which growth limitation switches from one element to another. They found that TER for C and P in many aquatic consumers is about 120–160, but varies among aquatic invertebrates with different P content. The C : P ratios of periphyton under the 300, 1000 and 1500 light conditions were greater than 160. Imbalances in N : P ratios between producers and consumers have the potential to limit the growth of consumers. However, herbivores are less affected by N : P ratios in periphyton because the ratios of aquatic consumers are generally similar to periphyton (Elser et al. 2000b; Liess & Hillebrand 2005). Thus, the growth rates of *G. chinensis* under the high-light conditions were regulated by phosphorus availability because the periphyton had high C : P ratios. However, consumers show a pronounced compensatory feeding response to low-quality food (Hillebrand & Matthiessen 2009). Still, compensatory feeding has an insignificant effect on snail growth rates when periphyton C : N and C : P ratios are very high (Fink & Von Elert 2006). The periphyton in our system might have had excessively high C : P ratios to compensate snail growth. On the other hand, snail growth was also suppressed under the lowest levels of light. This might have been caused by reduced food availability owing to the low light availability (Hill et al. 1995). However, high-intensity ultraviolet (UV) radiation can lead to higher grazing activity by snails in low-light conditions than in high-light conditions (Liess et al. 2009). Therefore, Liess & Lange (2011) concluded that the main limitation of snail growth is not food quality or quantity but rather UV exposure. However, the lights used in our experiment do not emit UV radiation, and

thus, the grazing activity of *G. chinensis* could not have been affected by irradiation. In addition, light affects snail growth by changing snail activity in eutrophic conditions (Liess & Lange, 2011). Hence, *G. chinensis* growth was more likely to be limited by food quality and quantity in our oligotrophic system than by behavioural suppression by light. Periphyton biomass significantly differed between cages with and without snails, but only under the lowest levels of light; no difference was observed between the other light conditions. This suggests that food resources were depleted in the lowest levels of light because of low periphyton production. In addition, because there was little periphyton biomass under the lowest levels of light, the periphyton layer was very thin. Because herbivore growth rates are affected by the energetic cost of searching for food (Charnov, 1976), the feeding efficiency of the snails might have been low because of the thinness of the periphyton layer under these conditions. Kuhara et al. (2000) measured periphyton biomass in the 200-m stretch in the upper part of Horonai stream and reported that periphyton biomass corresponded to that in the treatment of the lower light condition (50 and 300 $\mu\text{mol s}^{-1} \text{m}^{-2}$) in our system. Therefore, periphyton biomass in the field where *G. chinensis* lives falls within the range of periphyton in our experimental systems. In addition, many environmental characteristics in our experimental system (i.e. herbivore density, light intensity, current velocity and nutrient concentrations) were very similar to those observed in the natural environment. These results suggest that the LNH might hold true under field conditions.

Hill et al. (2010) published results that rejected the LNH, where snail (*Elimia clavaeformis* Lea) growth rates increased monotonically with increasing primary

production in their streams. They suggested that the major reason for this result was that the target streams represented carbon-limited environments because of the high density of herbivores, and they predicted that differences in herbivore density might influence the outcome of LNH predictions regarding herbivore growth rates. Herbivore density changes with differences in productivity, water current velocity, and interactions among other species in natural streams (Hawkins 1981; Downes et al. 1993; Mallory & Richardson 2005). In addition, while the LNH predictions regarding periphyton and herbivores are generally accepted for oligotrophic environments (Urabe et al. 2002; Fanta et al. 2010), negative correlations between light intensity and nutrient content rates in periphyton have been observed at even higher phosphorus concentrations ($25 \mu\text{g L}^{-1}$) in stream ecosystems (Fanta et al. 2010). Therefore, the growth rates of herbivores are limited under high-light conditions and this potentially occurs under a wide range of nutrient concentrations in natural streams in which herbivore density differs.

Effects of light conditions on snail reproduction

Our results showed that the absolute quantity of phosphorus in gonad tissues was maximised at an intermediate light intensity. Differences in phosphorus allocation could affect egg production and / or hatching rate; for example, in experiments in which crustacean zooplankton (*Daphnia*) were fed food with high C : N and C : P ratios, egg production decreased (Færøvig & Hessen 2003; Smith et al. 2009) and hatching rate decreased with decreasing phosphorus concentrations in the egg mass (Urabe & Sterner 2001). Some studies have examined the effects of the C : P ratios of producers on

herbivore reproduction; for example, copepods (*Cyclops abyssorum* Sars) in lakes restrain the production of crude eggs by decreasing their egg maturation rate when C : N and C : P ratios increase in phytoplankton (Ventura & Catalan 2005). In a study on the snail *Pomatopyrgus antipodarum*, individuals that fed on periphyton with a high C : P ratio matured later than individuals reared on algae with a low C : P ratio (Tibbets et al. 2010). Thus, differences in light intensity might have indirect effects on herbivore reproduction by changing the C : P ratios of producers in oligotrophic environments. Few studies have attempted to detect the effects of light on herbivore reproduction by assessing changes in C : N : P ratios, and no study has detected causal relationships among them (Sterner 1998). Therefore, this is the first study to suggest an indirect effect of light conditions on herbivore reproduction. However, our results imply that *G. chinensis* preferentially invests ingested nutrients into gonad tissues in P-limited environments. Færøvig & Hessen (2003) reported that there may be trade-offs in the allocations of C, N and / or P to somatic and reproductive tissues. Because producers have high C : P ratios in oligotrophic environments, like our study site, the quantity of nutrients ingested by herbivores is restricted (Fanta et al. 2010). For this reason, in environments where periphyton C : P ratios are elevated because of high light intensities, it is difficult for herbivores to ingest enough nutrients to invest in both growth and reproduction. Short-lived *G. chinensis* might have a plastic response for investing nutrients in reproduction that is adaptive in oligotrophic environments.

In summary, our results suggest that light conditions affected the growth rate and reproduction of a herbivore in an oligotrophic environment by changing periphyton C :

P ratios. Light conditions vary widely according to riparian conditions, and have an over-riding effect on stream ecosystems (Hill et al. 1995; Richardson & Danehy 2007). They may also govern ecosystem processes and functions, such as the flow of matter among trophic levels in oligotrophic environments, by changing primary production and food quality. However, the predictions of the LNH have still not been confirmed for other herbivores such as aquatic insects. In addition, it is still unclear whether the indirect effects of light intensity on natural environments involve complex interactions. Further investigations of a wide variety of herbivores and more complicated systems are needed in future.

Table 2-1. Condition of physical and chemical environment in an experimental channel and Horonai river. Parameters of Horonai river were measured in sampling plot of *G. chinensis*.

	Channels ($\pm$ SE)	Horoai rive ($\pm$ SE)
Water temperature ($^{\circ}$ C)	11.3 (0.26)	11.6 (0.35)
Current water speed (cm/sec)	8 (0)	10.1 (1.2)
Water depth (cm)	15 (0)	20.3 (1.3)
Inorganic nitrate (μ g/L)	16.4 (4.1)	20 (5.1)
Total phosphorus (μ g/L)	0.8 (0.19)	1.0 (0.26)

Table 2-2. The most parsimonious models for explaining the variance in periphyton production, CN ratio and CP ratio in periphyton. The modeling was conducted using a generalized linear model (GLM) with a stepwise selection based on Akaike's information criterion (AIC). Low light intensity indicates data from 50 $\mu\text{mol photons s}^{-1} \text{ m}^{-2}$ in the light condition. High light intensity indicates data from 300, 1000 and 1500 $\mu\text{mol photons s}^{-1} \text{ m}^{-2}$ in the light condition.

Response variable	Explanatory variable	Low light intensity				High light intensity			
		t value	Estimate $\pm$ SE	P value	AIC	t value	Estimate $\pm$ SE	P value	AIC
Diameter growth	Periphyton production	2.907	0.020 $\pm$ 0.007	0.010	10.603	-2.427	-0.004 $\pm$ 0.001	0.001	108.80
	Periphyton CN ratio	0.826	0.176 $\pm$ 0.213	0.420	17.970	1.043	0.108 $\pm$ 0.103	0.301	113.07
	Periphyton CP ratio	-0.958	-0.002 $\pm$ 0.002	0.352	17.993	-3.864	-0.0014 $\pm$ 0.001	<0.001	99.905

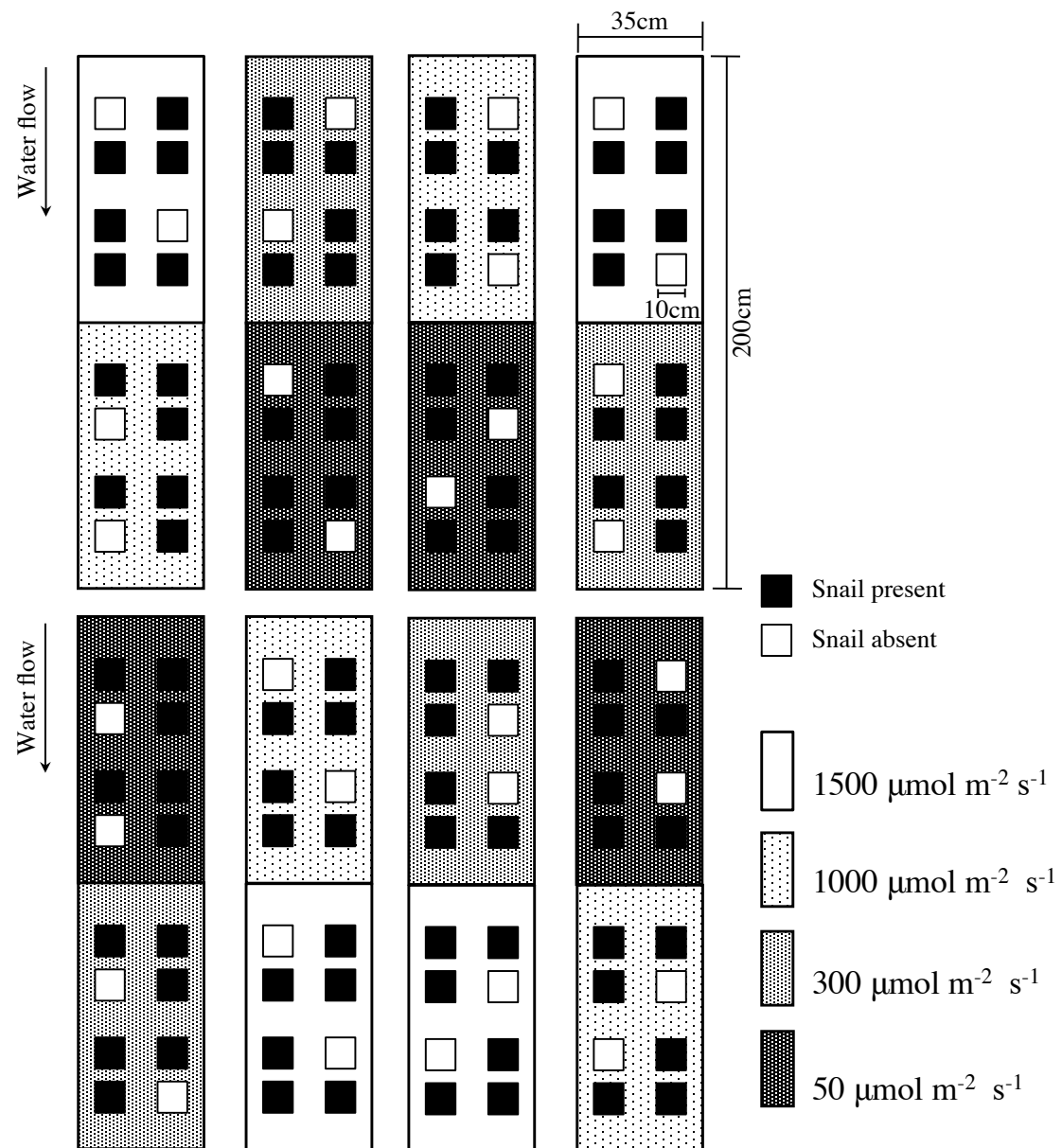


Fig. 2-1 Schematic diagram of experimental set-up.

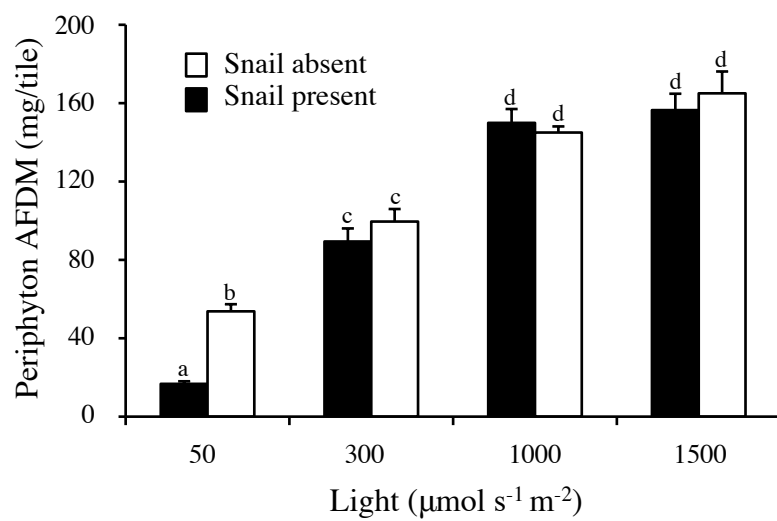


Fig. 2-2 Peiphyton biomass among four light conditions. Means and Standard errors ($\pm 1\text{SE}$) are shown. Significant differences ($P < 0.05$) among treatments are denoted by different letters.

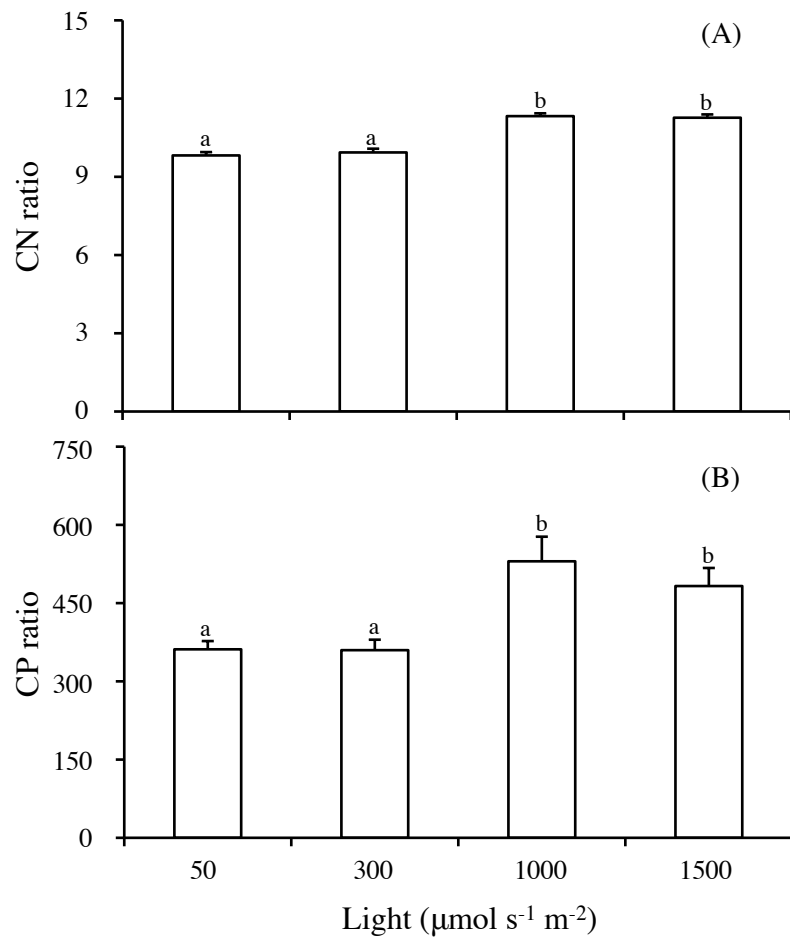


Fig. 2-3 CN ratio (A) and CP ratio (B) among four light conditions. Means and Standard errors ($\pm 1\text{SE}$) are shown. Significant differences ($P < 0.05$) among treatments are denoted by different letters.

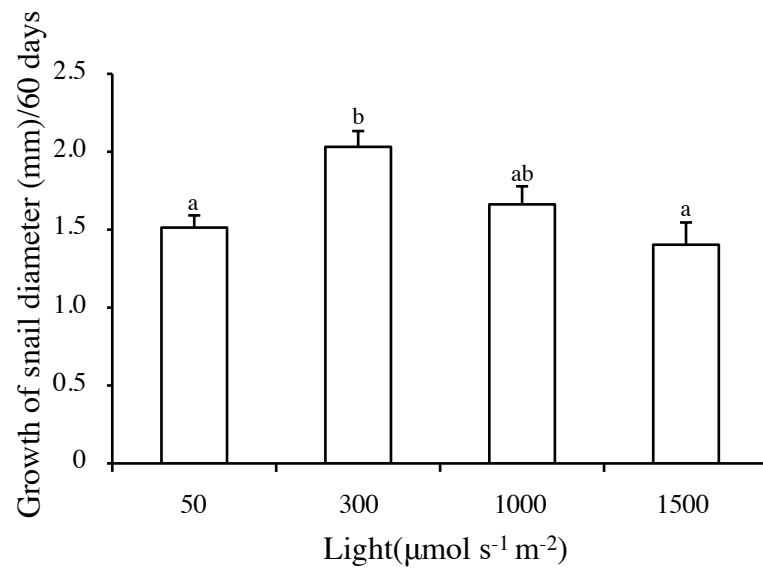


Fig. 2-4 Shell diameter growth rate among four light conditions. Means and Standard errors (+1SE) are shown. Significant differences ($P < 0.05$) among treatments are denoted by different letters.

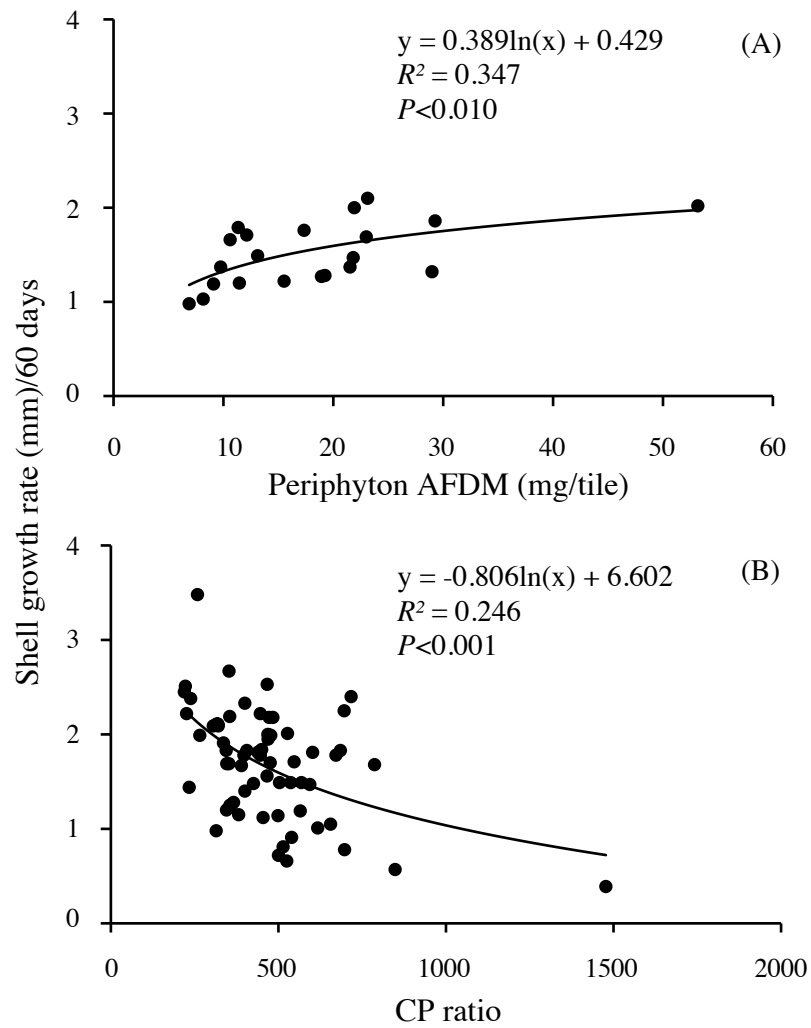


Fig. 2-5 Relationships between shell growth rate and periphyton AFDM (A) and CP ratio (B). Pannell (A) was drawn by using data from $50\mu\text{mol photons s}^{-1} \text{ m}^{-2}$, Pannell (B) was drawn by using data from 300, 1000 and $1500\mu\text{mol photons s}^{-1} \text{ m}^{-2}$.

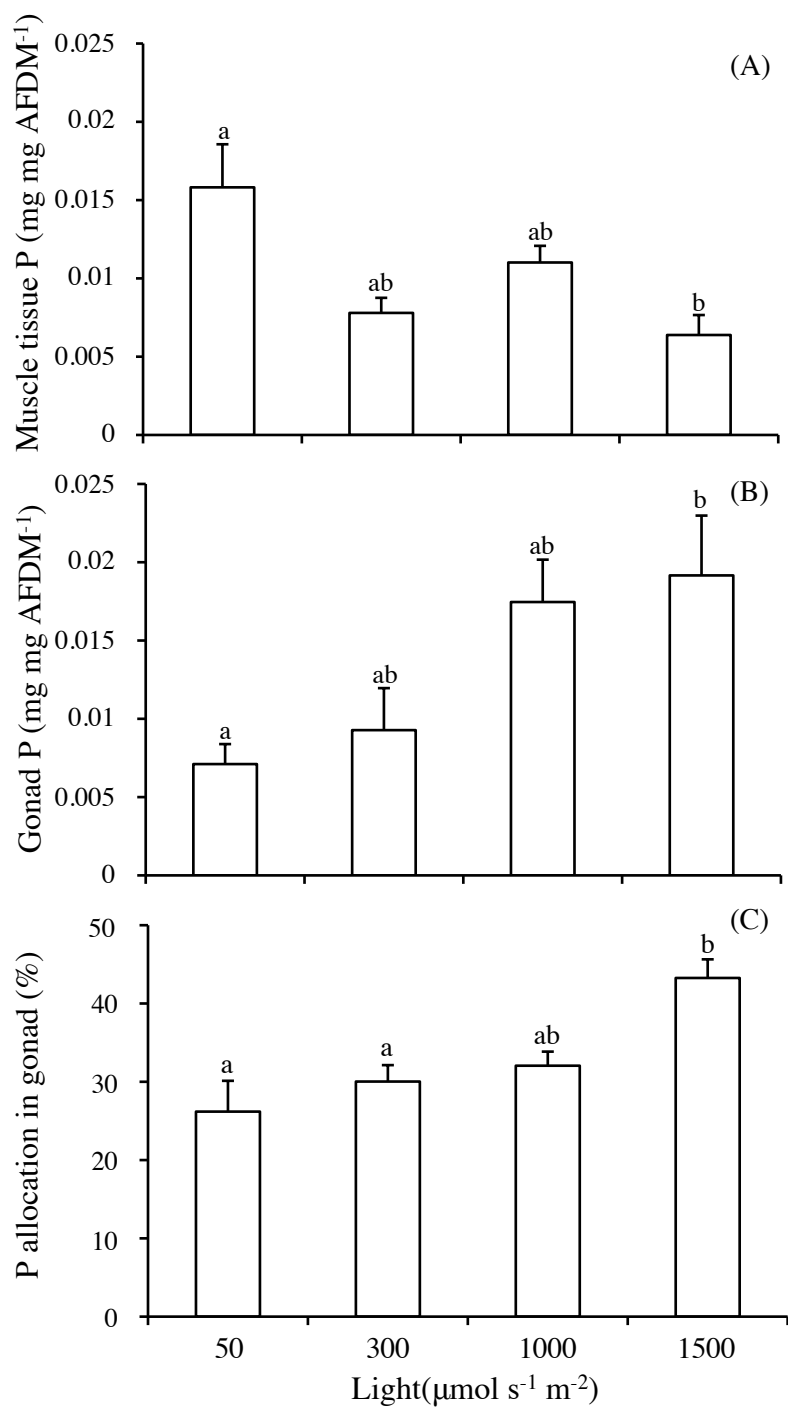


Fig. 2-6 Phosphorus concentration of muscle tissues (A) and gonad tissues (B), and allocation ratio of phosphorus in gonad tissue (C) among four light conditions. Means and Standard errors (+1SE) are shown. Significant differences ($P < 0.05$) among treatments are denoted by different letters.

Chapter 3

Calcium concentration in leaf litters of catchment vegetation affect abundance and survival of crustaceans in warm–temperate forests

INTRODUCTION

Movement of nutrients (e.g. nitrogen, phosphorus and various minerals) across ecosystem boundaries has a profound impact on community dynamics and interactions among species in recipient systems (Polis et al. 1997; Cross et al. 2006; Davis et al. 2010). Research on these subsidies has been frequently conducted in stream ecosystems, and alterations in terrestrial conditions, such as changes in vegetation and clear-cutting, alters the supply of subsidiary nutrients to stream ecosystems (Christopher et al. 2006; Fukuzawa et al. 2006; Tokuchi & Fukushima 2009). However, subsidiary calcium has received less attention, although it is an essential element for many animals. I considered that vegetation in a catchment area might alter the supply of subsidiary calcium, resulting in a change in calcium concentration in recipient streams.

Litter of members of the Cupressaceae has a higher concentration of calcium compared to other families (Kiilsgaard et al. 1987; Xue & Luo 2002; Baba et al. 2004). In this study, I focused on Japanese cedar (*Cryptomeria japonica*, Cupressaceae) because litter of this species has about 3% calcium (Xue & Luo 2002), which is more than three times higher than that of other taxa, such as fir (*Abies* spp.) and many broad-leaved tree species (Kiilsgaard et al. 1987; Baba et al. 2004; Reich et al. 2005). Japanese cedar plantations account for 12% of the total land area and 19% of the forested area in Japan (Forestry Agency 2011). Because forest soil organic matter is mainly produced from plant litter in the short term, the chemical properties of litter affect soil chemical properties (Reich et al. 2005). Indeed, the soil in Japanese cedar plantations has a calcium content that is three times higher than that in evergreen

broad-leaved forests in some parts of Japan (Tsutsumi 1987). Therefore, I hypothesized that the calcium concentration in stream water leached from soils might vary depending on catchment vegetation. In addition, *C. japonica* plantations are frequently clear-cut to harvest timber, which stops the supply of fresh litter containing abundant calcium, potentially inducing calcium depletion in the soil and streams.

I hypothesized that vegetation in a catchment affects the density and/or survival of aquatic crustaceans by altering the calcium concentration. Calcium is an essential element for many animals, particularly crustaceans, which are frequently dominant decomposers in freshwater systems (Macan 1961; Rukke 2002; Cairns & Yan 2009). Because crustaceans contain a large amount of calcium in their exoskeletons, they require calcium to support the body and protect it against physical damage. However, crustaceans lose a major portion of their total body calcium at each molt (Greenaway 1985; Wheatly 1999). After the molt, crustaceans must absorb adequate calcium to calcify their exoskeleton rapidly (Rukke 2002; Alstad et al. 1999; Hessen et al. 2000). As aquatic crustaceans extract calcium from the water via active branchial uptake (Wheatly 1999; Hammond et al. 2006), their uptake may be negatively affected in calcium-poor water. Calcium concentrations vary markedly among water bodies (Jeziorski et al. 2008), whereas the molt cycle duration of crustaceans is genetically fixed, regardless of *in situ* calcium levels (Rukke 2002; Hammond et al. 2006). Thus, even if the calcium concentration in a body of water decreases, crustaceans cannot delay molting. Therefore, the calcium concentration in a body of water is likely to impose

constraints on the growth and survival of crustaceans and affect their density and distribution (Hammond et al. 2006; Ashforth & Yan 2008; Strecker et al. 2008).

I examined the effect of Japanese cedar (*C. japonica*) plantations on aquatic invertebrate community structure, particularly on crustacean density and survival. I conducted field surveys and experiments in nine streams differing in catchment vegetation. I predicted that in a calcium-poor geographic condition: (1) calcium concentration is higher in streams of catchments dominated by *C. japonica* and lower in clear-cut and evergreen broad-leaved catchments and (2) crustacean density and survival are higher in streams of catchments dominated by *C. japonica* and lower in clear-cut and evergreen broad-leaved catchments.

METHODS

Study area

I conducted field surveys and a field experiment in May and June 2012 in nine fishless headwater streams of the Koza River running through the Wakayama Experimental Forest of Hokkaido University (33°40'N, 135°40'E; 428 ha) and surrounding private forests in southern Wakayama, Japan (Fig. 1). The geologic structure in this region consists of sandstone and mudstone formed during the middle Tertiary (Tateishi 1976). Because the soil is highly acidic and there is high annual rainfall (about 4000 mm), the environment is extremely poor in calcium (Kihira et al. 2005). The catchment areas of the streams are covered by a very thin soil layer, nearly exposing the base rock. Remnant natural evergreen broad-leaved forests are patchy, and Japanese cedar was

planted in much of the area beginning in the 1960s.

The study sites consisted of 30 m reaches in each of the nine fishless streams. Catchment areas of sites 1–3 were mostly composed of evergreen broad-leaved forest (EB sites), sites 4–6 consisted of Japanese cedar plantations (CP sites) and sites 7–9 were clear-cut (CC sites). Forests at the EB sites were dominated by *Quercus acuta*, *Q. myrsinaefolia*, *Q. sessilifolia*, *Neolitsea aciculata*, *Eurya japonica* and *Machilus thunbergii* (Wakayama Experimental Forest, unpublished data). The *C. japonica* trees at the CP sites were planted 29–81 years prior to this study (Table 3-1). The CC sites were logged 3–6 years prior to this study and were previously a Japanese cedar plantation (Table 3-1). The nine stream sites had very low flow over rocky substratum, ranging from 0.3–1.0 m in width and were generally less than 15 cm deep. Flow rate was not affected by the type of vegetation in the catchment area, and water quality at these sites was similar, except electrical conductivity, which indicates the amount of dissolved ions (Table 3-1).

Field survey

I monitored water temperature hourly during the experimental period using a temperature sensor with a logger (Tidbit v2 UTBI-001; Onset, Bourne, MA, USA) set at the headwaters of each stream. Other field sampling was conducted from 7 to 9 May 2012. At each site, I collected five water samples in polyethylene bottles (300 mL) to measure the concentrations of calcium, nitrogen and phosphorus. Ten samples of benthic invertebrates were collected over a 30-m reach at each site using a Surber net

sampler (25 × 25 cm quadrat) to establish the density and distribution of crustaceans. I collected 15 samples from the litter and soil layers using a core sampler (5 cm in diameter and 5 cm in height) to measure the soil calcium concentration in each water catchment area. I placed three litterfall traps (1 m²) at site 1 (EB) and site 4 (CP) on 7 May 2012 and collected the samples to measure the chemical properties of the litter.

Field experiment

I collected *Gammarus nipponensis* at CP sites 4 and 5 1 day before the experiment. On 9 May 2012, I placed 10 nylon cages (11 × 11 × 20 cm, 1 mm mesh) in each of the nine streams, and added 10 *G. nipponensis* to each cage. I placed *G. nipponensis* individuals from site 4 in five of the cages, and I placed *G. nipponensis* individuals from site 5 in the remaining five cages. All individuals used for the experiment were unsexed adults 8–10 mm in length. To estimate the relative importance of calcium in stream water and in litters for *G. nipponensis*, I also placed 5 g litter of *C. japonica* in all cages. The physical environment (i.e. water temperature, flow rate and water depth) at each experimental site were similar. I counted the number of surviving *G. nipponensis* in the cages at each site on 5 June 2012.

Sample processing

The benthic invertebrate samples were preserved in 99% ethanol and later identified to the lowest possible taxonomic level, usually genus or species. Water samples were filtered through glass filters (GF/C no. 1822; Whatman, Maidstone, Kent, UK) and then

frozen at -30°C for chemical analyses. Soil samples (litter layer and soil layer) were dried at 40°C for 48 h in a drying oven (NDO-450ND; Eyela, Tokyo, Japan). The litter layer samples were crushed using a blender (WB-1; Waring Products, New Hartford, CT, USA), and soil layer samples were sieved (<2 mm mesh) to remove coarse fragments. The crushed litter layer samples were ashed at 490°C for 2 h in an electric muffle furnace (KM-420; Advantec, Tokyo, Japan) and extracted with 1 M HCl at 80°C for 1 h. Dried soil layer samples were extracted with distilled water for 1 h. The litter and soil extracts were analysed for calcium concentration per unit dry mass (DM) using an inductively coupled plasma (ICP) atomic emission spectrometer (ICPE-9000; Shimadzu, Kyoto, Japan). The concentration of nitrate (NO_3^- and NO_2^-) and ammonium (NH_4^+) in the water samples was measured using standard methods (APHA, 2005), and concentrations of phosphorus and calcium were measured using an ICP atomic emission spectrometer. Litter samples collected from the litterfall traps were dried at 40°C for 48 h, sorted, and identified to species. Then, litter samples of each species were crushed using a blender, ashed at 490°C for 2 h in an electric muffle furnace and extracted with 1 M HCl at 80°C for 1 h. The extracts were analysed for calcium, phosphorus, potassium, magnesium, carbon and nitrogen concentration per unit DM using an ICP atomic emission spectrometer and a C/N analyzer (Sumigraph NC-900; Sumika Chemical Analysis Service, Osaka, Japan).

Statistical analysis

The physical and chemical environments in the streams (average water temperature,

total calcium, flow rate, pH, turbidity, electric conductivity, dissolved inorganic nitrogen and phosphorus concentration) were analysed using one-way analysis of variance (ANOVA) with catchment vegetation type as an independent variable, followed by post hoc comparisons using Tukey's method. Chemical properties of litter sample were also analysed using one-way ANOVA with litterfall trap as an independent variable, followed by post hoc comparisons using Tukey's method.

The abundance of invertebrates, calcium concentration in soil (litter layer and soil layer) and survival rate of *G. nipponensis* were fit to generalized linear mixed models with catchment vegetation type as a fixed factor and site identity as a random factor. Invertebrate abundance, total calcium in water and survival rate were assumed to follow Poisson, normal, and binomial distributions, respectively. The effect of total calcium and NO_3^- in stream water on survival rate was evaluated by logistic regression with site identity as a random factor. The statistical significance of the effect of the fixed factor in each model was evaluated by a likelihood ratio test ($\alpha = 0.05$). When the effect of vegetation type was significant, post hoc comparisons using likelihood ratio tests were conducted for all three pairs of vegetation types with a significance level adjusted by Bonferroni's method ($\alpha = 0.05/3$). Because the origins (sites 4 and 5) of *G. nipponensis* individuals did not significantly affect their survival rates (likelihood ratio test: $\chi^2 = 0.100$, $P > 0.75$), the survival rates were not separately analysed with their origins.

I performed canonical correspondence analysis (CCA) to explore the relationships among species composition and catchment vegetation types and physical

and chemical properties of the stream water. Prior to analyses, four extremely rare taxa (<0.03% in total abundance) were removed, and abundance data for each species were standardized to unit variance. Before conducting the CCA ordination, we selected the most important explanatory variables from all physical and chemical properties of stream water by forward stepwise selection based on Akaike's information criteria and Monte Carlo permutation tests. Electric conductivity was excluded from the forward selection as it correlated highly with total water calcium ($r = 0.871$, $P = 0.002$). The significance of the CCA ordination axes was tested using Monte Carlo permutation tests. I also evaluated the variation explained by each explanatory variable using the variation partitioning method (Borcard et al., 1992). I calculated the conditional inertia in CCA by choosing one variable as a covariable, which indicated the variation in species composition explained by that variable, but it could include effects of other correlated variables. Second, I obtained the constrained inertia in CCA choosing the other variables, indicating the variation explained by that one variable independently of the others.

All statistical analyses were conducted with R Version 2.9.2. software (R Development Core Team, 2009).

RESULTS

Field survey

Total calcium (ANOVA: $F = 31.44$, $df = 2$, $P < 0.001$), electric conductivity (ANOVA: $F = 39.71$, $df = 2$, $P < 0.001$) and NO_3^- (ANOVA: $F = 10.22$, $df = 2$, $P = 0.011$) of

stream water differed among catchment vegetation types, whereas the other chemical and physical properties did not differ (Table 3-1). Total calcium and NO_3^- at CP sites were significantly higher than EB and CC sites, and differences among EB and CC sites were not significant (Table 3-1). Electric conductivity at CP and CC sites were significantly higher than EB sites, but no differences were noted among CP and CC sites (Table 3-1). Water at the CP sites had three to four times higher total calcium than that at the EB sites, although NO_3^- at the CP sites was only 1.5 times higher than that at the EB sites. Because all NO_2^- concentrations were below the detection limit, values are not given in Table 3-1. Soil calcium concentration also varied among catchment vegetation types (Fig. 3-2). Calcium concentrations in the litter layer differed among the catchment vegetation types (likelihood ratio test: $\chi^2 = 15.79$, $\text{df} = 2$, $P < 0.001$), and the litter layer at the CP sites had about three times higher concentration of calcium than those at the EB sites ($P < 0.002$; Fig. 3-2a). Water-extractable calcium concentrations in the soil layer also differed among catchment vegetation types (likelihood ratio test: $\chi^2 = 29.21$, $\text{df} = 2$, $P < 0.001$), and CP sites had three to four times higher calcium concentrations than those at EB and CC sites ($P < 0.002$; Fig. 3-2b), whereas calcium concentrations at the EB and CC sites did not differ ($P = 0.29$; Fig. 3-2b). Based on the litter-trap samples from sites 1 and 4, the calcium concentrations in litter differed considerably among species (ANOVA: $F = 14.57$, $\text{df} = 6$, $P < 0.001$), and the concentration in *C. japonica* litter was about three times higher than that of dominant evergreen broad-leaved trees (Table 3-2).

The primary consumer and decomposer communities in the nine streams

comprised 18 taxa, and the predator community was dominated by two genera (*Sweltsa nikkoensis* and *Oyamia* sp.) of invertebrates. The dominant taxa (>5% in total abundance) consisted of the crustacean *G. nipponensis*; three mayflies (Ephemeroptera), *Baetis* sp., *Cinygmula* sp. and *Paraleptophlebia* sp., and chironomid midges (Table 3-3). Another crustacean, the Japanese freshwater crab *Geothelphusa dehaani*, was also found, although densities were low. The density of *G. nipponensis* differed among streams in catchments with different vegetation types (likelihood ratio test: $\chi^2 = 12.48$, $df = 2$, $P = 0.002$). This crustacean occurred at very high densities at CP sites, but it was collected in low numbers at the EB and CC sites ($P < 0.003$; Fig. 3-3a). In addition, the density of *G. dehaani* also differed among the catchment vegetation types (likelihood ratio test: $\chi^2 = 21.31$, $df = 2$, $P < 0.001$). *G. dehaani* density was higher at CP than that at EB sites ($P < 0.001$; Fig. 3-3b), whereas densities at CP and CC sites were similar ($P = 0.37$; Fig. 3-3b). The benthic decomposer communities at CP sites were markedly dominated by *G. nipponensis*, whereas few decomposers were collected at the EB and CC sites. *Baetis* sp. and *Cinygmula* sp. were the dominated grazers at all sites, and *Baetis* sp. density was only significantly lower at CP sites ($P = 0.001$); all other taxa were similar across catchment vegetation types (Table 3-4). In addition, the total density of invertebrates varied greatly, depending on *G. nipponensis* density.

Forward selection showed that water chemistry explained significant variation in invertebrate community composition among the nine stream sites. The first four axes and axes one and two explained 38.2 %, 33.8% and 2.9%, respectively, of the variation in species composition (Monte Carlo permutation test: $P < 0.05$). The CCA ordination

showed that the community structure of stream invertebrates clearly varied with catchment vegetation type (Fig. 3-4). In particular, community composition at the CP sites was distinctively different from that at the EB and CC sites along with the first CCA axis, which was positively correlated with total calcium and NO_3^- and negatively correlated with total phosphorus. The compositional difference between the EB and CC sites was smaller than that between the CP and EB or CC sites. *G. nipponensis* had a large positive score on the first axis, and its high abundance characterized the CP community, whereas most other taxa had negative scores on the first axis, indicating abundance peaks in the middle of EB and CP. Results of variation partitioning indicated that total calcium and NO_3^- explained a large proportion of the variation in taxonomic composition (Table 3-5).

Field experiment

Sites 4, 7 and 8 dried up during the experimental period; thus, no data were available from these sites. The survival rate of *G. nipponensis* differed among the catchment vegetation types (likelihood ratio test: $\chi^2 = 25.18$, $\text{df} = 2$, $P < 0.001$). Furthermore, the survival rate of *G. nipponensis* increased with increasing total calcium in the stream (likelihood ratio test: $\chi^2 = 22.57$, $\text{df} = 1$, $P < 0.001$; Fig. 3-5).

DISCUSSION

This is the first study to show that terrestrial vegetation may affect community structure of benthic invertebrates by altering subsidiary calcium in a body of water. The results of

our field survey showed that total calcium was three to four times higher in streams flowing through catchments dominated by *C. japonica* plantations compared to those in evergreen broad-leaved forest and clear-cut areas (supporting prediction 1); densities of *G. nipponensis* and *G. dehaani* were higher in streams flowing through *C. japonica* plantations (supporting prediction 2); benthic invertebrate community structure varied with catchment vegetation types and total calcium in stream water was the most important environmental variable explaining the variation in community composition. The field experiment showed that survival of *G. nipponensis* was higher in calcium-rich streams flowing through *C. japonica* plantations (supporting prediction 2).

Calcium concentrations in leaf litter, soil and stream water were three to four times higher at CP sites than at EB sites, suggesting that catchment vegetation type affects total calcium in streams. Studies conducted at the Hubbard Brook Experimental Forest in the northeastern United States indicated that adding CaSiO_3 to a catchment area increases calcium concentrations in soil and stream water (Juice et al. 2006; Minocha et al. 2010; Nezat et al. 2010) and alters the community structure of terrestrial snails (Skeldon et al. 2007). These results support our finding that subsidiary calcium applied through *C. japonica* litter increased the calcium concentration in streams flowing through the study area. Although why *C. japonica* litter has a high calcium content is not yet understood, some fruit tree species can take in calcium-containing fertilizer through stomata (Schlegel & Schönherr 2002; Hossain & Ryu 2009). If *C. japonica* also has such a trait, Japanese cedar trees might be able to obtain calcium from rainfall, fog or aerosols.

Our results showed that the density and survival of *G. nipponensis* were correlated with catchment vegetation. Zehmer et al. (2002) demonstrated that the distribution of the crustacean *Gammarus pseudolimnaeus* may be affected by the calcium concentration in stream water, but they did not examine which factors caused the variation in calcium concentration. Several studies have shown that aquatic gammarid amphipods are unable to survive below a certain threshold concentration of calcium (25–125 $\mu\text{mol L}^{-1}$) (Rukke 2002; Zehmer et al. 2002; Wright 1980). The low density and poor survival of *G. nipponensis* at the EB sites suggest that total calcium of stream water in the evergreen broad-leaved forest may have been below the necessary threshold for this species. Total calcium in stream water varies seasonally due to fluctuations in discharge (Christopher et al. 2006; Tokuchi & Fukushima 2009). However, Iwayama (unpublished data) showed bimestrial calcium concentrations at site 1, 4 and 5 in 2010, similar to our findings: total calcium concentration at site 1 (EB) was low throughout the year (20–60 $\mu\text{mol L}^{-1}$), whereas total calcium at sites 4 and 5 (both CP) was greater than 100 $\mu\text{mol L}^{-1}$. Therefore, total calcium at the EB sites might be extremely low, whilst concentrations at CP sites were likely high throughout the year. In addition, our results showed that invertebrate community composition was related to vegetation and forest management practices (plantation and logging of cedar) and water chemistry. In particular, total calcium and NO_3^- were strong predictors of community composition. However, total calcium in stream water was more variable than dissolved NO_3^- , and gammarid amphipods have a calcium lethal threshold point (Rukke 2002; Zehmer et al. 2002; Wright 1980). The variation in community composition along with

the gradient in water chemistry (including total calcium) could be caused by the drastic changes in abundance of *G. nipponensis*. In fact, when *G. nipponensis* was excluded from the analysis, the first four axes and axes one and two of the CCA explained only 14.0%, 6.9% and 3.8%, respectively, of the variation in taxonomic composition. These results show that total calcium in the stream water had the greatest impact on the variation in stream invertebrate communities at our study sites by altering the abundance of *G. nipponensis*. Calcium ions must be leached out with an inorganic anion, and NO_3^- might be considered a counterion (Christopher et al. 2006). Therefore, despite the fact that the nitrogen content of the leaf litter did not differ among the *C. japonica* and evergreen broad-leaved forests, NO_3^- showed a similar pattern to calcium among streams. The first axis of CCA correlated with NO_3^- and total calcium, and NO_3^- had large effects on community composition at our sites.

Recent studies have shown that the abundance or density of gammarid amphipods is significantly greater in streams that drain Japanese cedar plantations than in those that drain deciduous broad-leaved forests (Hisabae et al. 2010; Inoue et al. 2012; Sakai et al. 2013). These authors argue that greater invertebrate abundances/densities are the result of *C. japonica* litter providing a predictable food resource for shredders, due to its long period of abscission, slow breakdown and low dispersal. However, these studies did not measure total calcium and therefore could not include this variable as a potential predictor of gammarid abundance between the two forest types. Our results show that one must consider calcium availability when studying density and survival of gammarid amphipods. Further study is needed to verify

the relative importance of calcium availability for invertebrate densities as well as how litter quality varies with forest type.

I considered gammarid amphipods might ingest calcium directly from plant litter, which is their main food resource. However, calcium is usually in a chelated form in plant tissue, making it difficult for animals to utilize (Nakata & McConn 2007). In addition, *C. japonica* litter is generally regarded as an unsuitable food for invertebrates because of its toughness and low nutritive quality (Hisabae et al. 2010). Thus, dissolved calcium is more important for crustaceans than the calcium content in litter deposited on a streambed. The rate of calcium uptake by crustaceans after molting is also dependent on the pH of the water (Malley 1980). However, because pH in the study streams was similar it not have a significant effect on the density or survival of *G. nipponensis*.

The density of *G. nipponensis* was very low not only at the EB sites but also at the CC sites. Total calcium in water at the CC sites was lower than that at the CP sites, suggesting that calcium in streams of clear-cut areas is below the threshold necessary for this species to survive. Therefore, logging of *C. japonica* appears to influence aquatic crustacean populations within the catchment area by altering total calcium in stream water. The drastic decrease in *G. nipponensis* by logging also affected the invertebrate community structure, similar to that at EB, although the community structure at CC and EB was still distinctively different. Gammarid amphipods are not tolerant of physical disturbances, such as flash floods, debris flows or drought (Inoue et al. 2012; Kobayashi et al. 2013). Such severe physical disturbances in clear-cut areas might partly affect the density and survival of *G. nipponensis* and community

composition of the invertebrates.

Acid deposition has depleted calcium in soil and freshwater systems worldwide (Jeziorski et al. 2008; Federer et al. 1989; Likens et al. 1996). This depletion of calcium in turn causes a decrease in pH, which has many adverse environmental effects. For example, the soil nutrient cycle is tightened and toxic substances (e.g. aluminium) are released from the soil to aquatic ecosystems (Likens et al. 1996; Driscoll et al. 2001). In this context, our results suggest that the intensity of these adverse effects might be altered by terrestrial vegetation and management practices. Although *C. japonica* is an endemic species in Japan, the litter of other widely distributed members of Cupressaceae, such as *Chamaecyparis* and *Sequoiadendron*, also have calcium contents comparable to that of *C. japonica* (Kiilsgaard et al. 1987; D'Amore et al. 2009). Many previous studies (e.g. Likens et al. 1998; Neal et al. 1992; Lawrence et al. 1999) conducted monitoring of calcium concentration in streams in the United States; calcium concentrations in our sites were similar. Additional research is needed to confirm the effect of terrestrial vegetation and its management on freshwater systems through alterations in total stream calcium in other areas of the world including calcium-depleted ecosystems.

Although total calcium explained density and survival of crustaceans in our study, other factors might be also important. To better test the importance of calcium future studies should focus on manipulating calcium concentration in stream and laboratory experiment. Furthermore, studies of how calcium movement can be altered

by plantation of *C. japonica* including atmospheric and soil biogeochemical processes are needed.

Since calcium concentration in freshwater affects not only crustaceans but also freshwater snails (e.g. Huryn et al. 1995), catchment vegetation may also be an important predictor of the density or survival of freshwater snails. Our results show that catchment vegetation type and management practices can alter stream invertebrate communities by altering total calcium, which may in turn affect community dynamics and functional ecosystem processes. Tree plantations also affect soil invertebrate communities (Reich et al. 2005; Tsukamoto & Sabang 2005), as reported for *C. japonica* plantations (Watanabe 1973; Touyama & Nakagoshi 1994; Ikeda et al. 2005). However, no study has specifically examined the effect of plantations on soil invertebrate communities through alterations in calcium concentrations. Soil calcium concentration is a limiting factor for terrestrial crustaceans and earthworms, which are important decomposers (Springett & Syers 1984). Therefore, our findings might be applicable to terrestrial communities as well, and they highlight the role of vegetation change as a driver of regional biogeochemistry.

Table 3-1 Catchment vegetation and physicochemical conditions (mean $\pm$ 1 SE) of stream water at each site. NA means below measurable limit. Significant differences among vegetation types are denoted by different letters in the last line (Tukey test, $P < 0.05$).

	Evergreen broad-leaf forest (EB)			Cedar plantation (CP)			Clear-cut plantation (CC)			Significant differences among vegetation types
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	
Stand age (year)	< 100	< 100	< 100	29 - 54	29 - 81	< 50	3	6	6	
or Passed age from clear-cutting										
Altitude of water catchment area (m)	380 - 680	380 - 800	180 - 500	420 - 680	380 - 680	240 - 660	480 - 720	180 - 320	280 - 740	
Catchment area (km ²)	0.28	0.79	0.44	0.28	0.24	0.42	0.90	0.15	0.56	
Water temperature (°C)	13.1 - 17.6	13.6 - 17.9	13.8 - 18.1	14.0 - 18.1	14.1 - 18.3	13.5 - 18.1	13.5 - 17.9	13.7 - 18.7	14.0 - 19.2	EB ^a CP ^a CC ^a
Flow rate (m ³ sec ⁻¹)	0.83	1.31	1.25	1.84	1.11	0.92	1.48	0.69	1.05	EB ^a CP ^a CC ^a
pH	7.01	6.98	6.8	7.07	7.19	7.00	6.77	7.04	7.12	EB ^a CP ^a CC ^a
Turbidity (NTU)	0	0	0	0	0	0	0	0	0	EB ^a CP ^a CC ^a
Electric conductivity (S m ⁻¹)	0.9	1.5	1.2	2.5	2.4	2.6	2.2	2.2	2.1	EB ^a CP ^b CC ^b
NO ₃ ⁻ (μmol L ⁻¹)	4.22 (0.24)	4.32 (0.31)	4.36 (0.36)	6.34 (0.35)	6.21 (0.29)	5.89 (0.57)	4.11 (0.34)	4.28 (0.31)	5.71 (0.48)	EB ^a CP ^b CC ^a
NH ₄ ⁺ (μmol L ⁻¹)	0.82 (0.14)	0.21 (0.08)	0.26 (0.08)	0.32 (0.09)	0.26 (0.10)	0.33 (0.08)	0.32 (0.09)	0.34 (0.06)	0.21 (0)	EB ^a CP ^a CC ^a
Total phosphorus (nmol L ⁻¹)	1.6 (0.5)	1.8 (1.0)	1.6 (0.9)	NA	1.6 (0.6)	1.6 (0.8)	1.5 (0.8)	1.7 (0.7)	NA	EB ^a CP ^a CC ^a
Total calcium (μmol L ⁻¹)	15 (1.25)	20 (1.57)	17 (2.71)	49 (5.55)	59 (3.18)	68 (4.11)	35 (3.43)	27 (4.10)	37 (1.96)	EB ^a CP ^b CC ^a

Table 3-2 Elemental concentration (mean $\pm$ 1 SE) in leaf litters of dominant tree species.

Leaf litter of evergreen broad-leaved trees and *Cryptomeria japonica* were obtained by litter-fall traps at sites 1 and 4. Significant differences among vegetation types are denoted by different letters (Tukey test, $P < 0.05$).

	Concentration (mg g ⁻¹)						P
	Ca	K	Mg	C	N		
<i>Quercus acuta</i>	15.14 (4.67) ^a	5.90 (0.54) ^a	1.01 (0.32) ^a	525.75 (11.46) ^a	14.71 (2.26) ^a	0.56 (0.30) ^a	
<i>Quercus myrsinaefolia</i>	12.55 (4.05) ^a	6.93 (0.748) ^a	1.38 (0.14) ^a	514.17 (5.10) ^a	16.06 (2.22) ^a	0.31 (0.071) ^a	
<i>Neolitsea aciculata</i>	8.04 (1.55) ^a	6.49 (0.15) ^a	1.43 (0.42) ^a	538.27 (6.41) ^a	12.02 (2.40) ^a	0.35 (0.13) ^a	
<i>Quercus sessilifolia</i>	13.81 (2.34) ^a	6.27 (1.01) ^a	1.27 (0.23) ^a	512.83 (9.17) ^a	11.22 (2.66) ^a	0.12 (0.09) ^a	
<i>Eurya japonica</i>	12.61 (2.90) ^a	9.44 (1.42) ^a	1.62 (0.66) ^a	494.29 (16.24) ^a	10.88 (3.33) ^a	0.39 (0.065) ^a	
<i>Machilus thunbergii</i>	8.88 (4.34) ^a	6.48 (1.08) ^a	1.87 (0.58) ^a	526.76 (12.11) ^a	11.25 (0.79) ^a	0.40 (0.24) ^a	
<i>Cryptomeria japonica</i>	34.41 (4.23) ^b	2.42 (0.14) ^b	1.37 (0.62) ^a	493.04 (13.76) ^a	14.71 (3.91) ^a	0.56 (0.26) ^a	

Table 3-3 Abundance (mean $\pm$ 1 SE) of benthic invertebrates at each site.

	Abundance (no. m ⁻²)								
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9
Crustaceans									
<i>Gammarus nipponensis</i>	1.6 (1.6)	20.8 (20.8)	0 (0)	1422.4 (330.0)	1108.8 (307.9)	1265.6 (286.0)	11.2 (7.9)	11.2 (9.6)	191.4 (46.3)
<i>Geothelphusa dehaani</i>	3.2 (2.1)	4.8 (2.2)	3.2 (2.1)	0 (0)	17.6 (5.6)	25.6 (4.9)	19.2 (5.7)	17.6 (5.0)	20.8 (4.8)
Ephemeroptera									
<i>Cinygmula</i> sp.	5.3 (3.4)	230.4 (54.8)	228.8 (39.8)	109.8 (28.7)	409.6 (79.9)	198.4 (38.0)	158.6 (35.6)	178.6 (46.2)	418.0 (76.9)
<i>Baetis</i> sp.	378.6 (54.6)	462.4 (131.5)	409.6 (96.6)	388.3 (101.2)	97.6 (26.3)	196.8 (47.2)	384.0 (40.3)	286.8 (31.2)	384.0 (102.6)
<i>Paraleptophlebia</i> sp.	106.6 (28.2)	27.2 (12.2)	25.9 (11.2)	29.8 (17.8)	88.0 (27.0)	83.2 (15.0)	49.8 (18.8)	54.6 (11.4)	208.0 (56.1)
<i>Ephemera japonica</i>	1.6 (1.6)	33.6 (13.8)	22.4 (9.3)	27.2 (10.7)	17.6 (14.2)	14.4 (2.9)	11.2 (4.2)	14.4 (4.4)	1.6 (1.6)
<i>Bleptus fasciatus</i>	0 (0)	3.2 (2.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Trichoptera									
<i>Stenopsyche marmorata</i>	3.2 (2.1)	1.6 (1.6)	3.3 (3.2)	4.8 (2.4)	4.8 (2.4)	4.8 (3.4)	0 (0)	0 (0)	0 (0)
<i>Goerodes</i> sp.	6.4 (3.54)	3.2 (2.1)	20.8 (10.4)	0 (0)	0 (0)	24.0 (14.4)	0 (0)	0 (0)	0 (0)
<i>Glossosoma</i> sp.	0 (0)	1.6 (1.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Plecoptera									
<i>Oyamia</i> sp.	17.6 (9.9)	27.2 (8.9)	9.6 (6.4)	19.2 (6.2)	1.6 (1.6)	48.0 (23.2)	17.6 (7.3)	11.2 (6.3)	22.4 (9.3)
<i>Sweltsa nikkoensis</i>	4.8 (4.8)	44.8 (19.2)	24.0 (12.7)	0 (0)	3.2 (3.2)	3.2 (3.2)	3.2 (3.2)	0 (0)	0 (0)
Diptera									
Chironomidae spp.	472.0 (136.8)	523.2 (255.2)	435.2 (111.2)	215.6 (143.9)	412.8 (97.4)	568.0 (59.0)	486.0 (96.8)	398.4 (68.2)	416.2 (149.9)
<i>Tipula</i> sp.	25.6 (15.3)	12.8 (6.7)	6.4 (3.5)	0 (0)	32.0 (8.3)	1.6 (1.6)	0 (0)	0 (0)	0 (0)
Simuliidae spp.	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.6 (1.6)	0 (0)	0 (0)	0 (0)
Coleoptera									
<i>Mataeopsephus maculatus</i>	6.4 (3.5)	4.8 (2.4)	0 (0)	4.8 (2.4)	35.2 (14.9)	0 (0)	22.4 (14.2)	8.0 (4.3)	20.8 (9.8)
Elmidae	12.8 (5.7)	1.6 (1.6)	14.4 (6.5)	14.4 (6.9)	8.0 (6.4)	33.6 (13.4)	12.8 (5.7)	30.4 (9.7)	56.0 (23.8)
Neuroptera									
<i>Protohermes grandis</i>	0 (0)	0 (0)	1.6 (1.6)	0 (0)	0 (0)	1.6 (1.6)	0 (0)	0 (0)	0 (0)
Total	1069.3 (144.3)	1401.6 (412.0)	1164.8 (182.6)	2679.8 (315.2)	2030.4 (454.4)	2436.8 (182.6)	1262.4 (220.3)	1181.8 (218.3)	1368.4 (186.2)

Table 3-4 Relationships between abundance of each invertebrate species and catchment vegetation types. The likelihood ratio test was used to test the difference in deviance between the selected model and the null model. Significant differences among vegetation types are denoted by different letters in the last line (post-hoc pairwise likelihood ratio tests, $P < 0.05/3$).

	Results of likelihood ratio test			
	<i>df</i>	χ^2	<i>P</i>	Significant differences among vegetation types ($P < 0.05/3$)
Crustaceans				
<i>Gammarus nipponensis</i>	2	12.48	**	EB ^a CP ^b CC ^a
<i>Geothelphusa dehaani</i>	2	21.31	***	EB ^a CP ^b CC ^b
Ephemeroptera				
<i>Cinygmula</i> sp.	2	3.85	n.s.	
<i>Baetis</i> sp.	2	11.71	**	EB ^a CP ^b CC ^{ab}
<i>Paraleptophlebia</i> sp.	2	1.01	n.s.	
<i>Ephemera japonica</i>	2	1.87	n.s.	
<i>Bleptus fasciatus</i>	2	0.00	n.s.	
Trichoptera				
<i>Stenopsyche marmorata</i>	2	5.81	n.s.	
<i>Goerodes</i> sp.	2	1.86	n.s.	
<i>Glossosoma</i> sp.	2	0.00	n.s.	
Plecoptera				
<i>Oyamia</i> sp.	2	0.00	n.s.	
<i>Sweltsa nikkoensis</i>	2	5.43	n.s.	
Diptera				
Chironomidae	2	0.42	n.s.	
<i>Tipula</i> sp.	2	2.68	n.s.	
Simuliidae	2	0.00	n.s.	
Coleoptera				
<i>Mataeopsephus maculatus</i>	2	1.98	n.s.	
Elmidae	2	3.70	n.s.	
Neuroptera				
<i>Protohermes grandis</i>	2	0.00	n.s.	

Table 3-5 Variation of stream invertebrate community composition explained by each environmental variable evaluated by partial canonical correspondence analyses (pCCAs).

Environmental variable	Variation explained (%)
Calcium concentration	26.18
NO ₃ ⁻	32.27
NH ₄ ⁺	3.81
Total phosphorus	6.92

Proportion of conditional inertia in total inertia in pCCA partialling out the one variable.

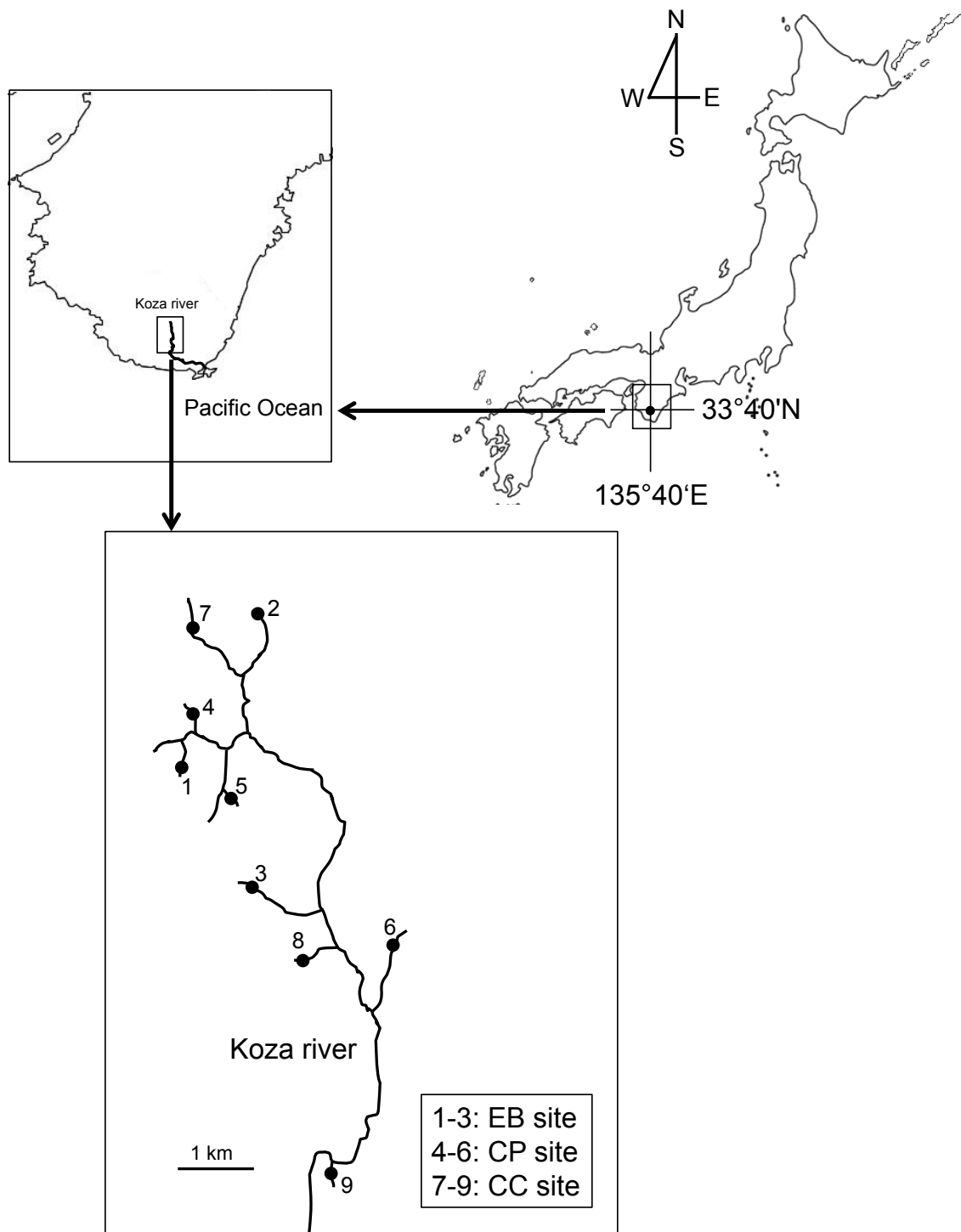


Fig. 3-1 Locations of the nine study streams. Streams 1-3 were in evergreen broad-leaved forests (EB), 4-5 were in cedar plantations (CP) and 7-9 were in clear-cut cedar plantations (CC).

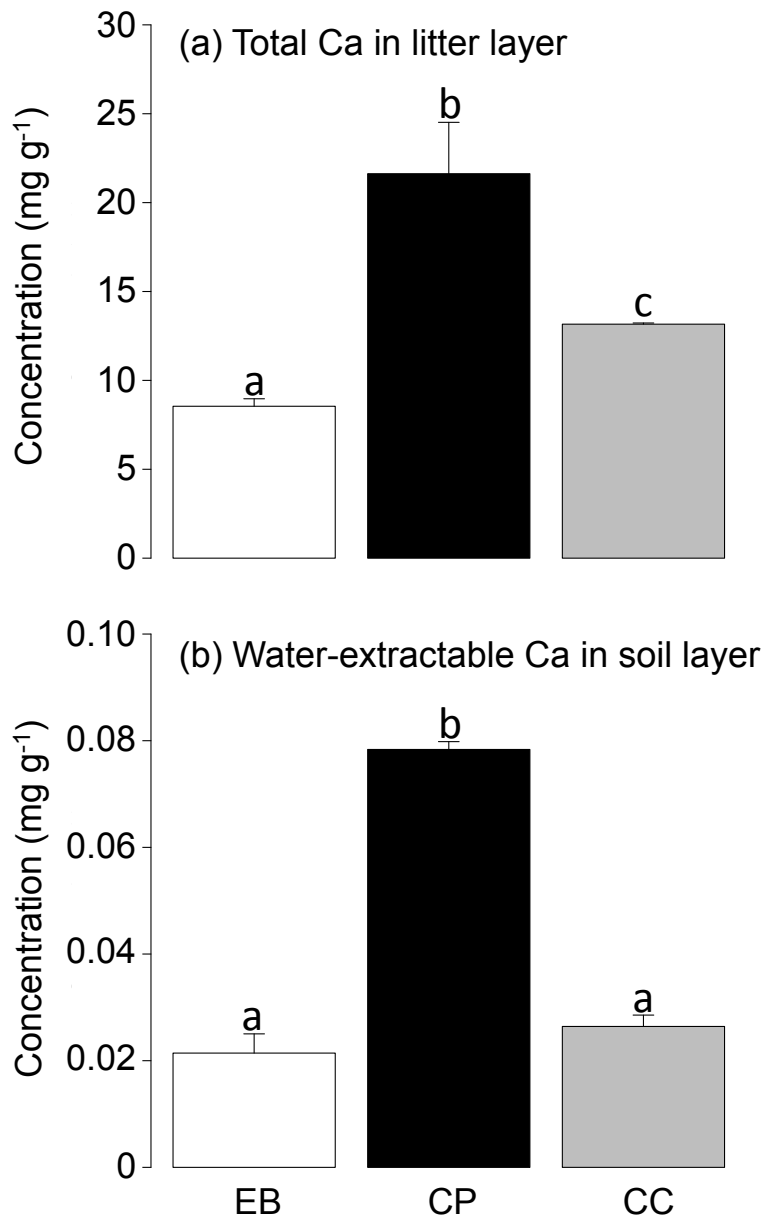


Fig. 3-2 Concentrations (mean $\pm$ 1 SE) of (a) total calcium in litter layer and (b) water-extractable calcium in soil layer in each catchment vegetation types. White, black and grey bars indicate evergreen broad-leaved forests (EB), cedar plantations (CP) and clear-cut cedar plantations (CC), respectively. Significant differences among vegetation types are denoted by different letters (post-hoc pairwise likelihood ratio tests, $P < 0.05/3$).

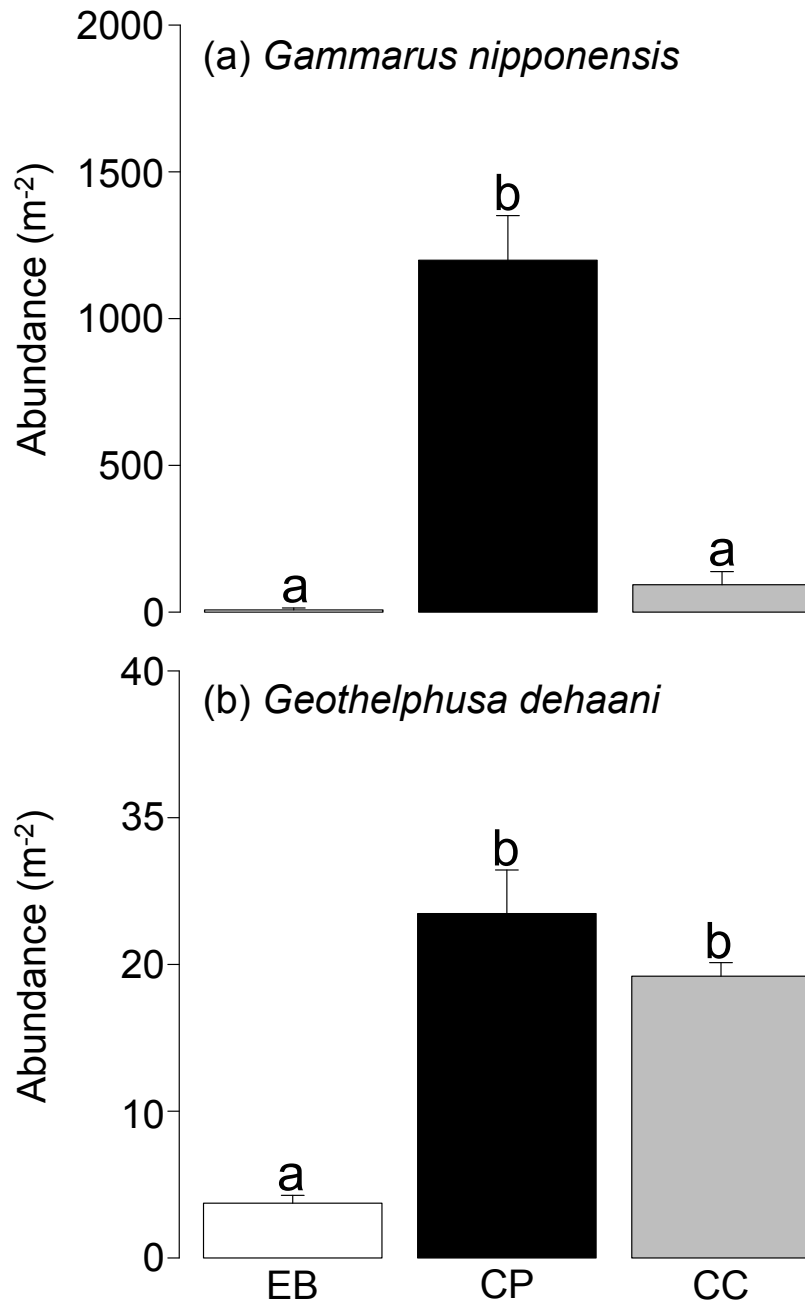


Fig. 3-3 Abundance (mean $\pm$ 1 SE) of (a) *Gammarus nipponensis* and (b) *Geothelphusa dehaani* in each catchment vegetation types. White, black and grey bars indicate evergreen broad-leaved forests (EB), cedar plantations (CP) and clear-cut cedar plantations (CC), respectively. Significant differences among vegetation types are denoted by different letters (post-hoc pairwise likelihood ratio tests, $P < 0.05/3$).

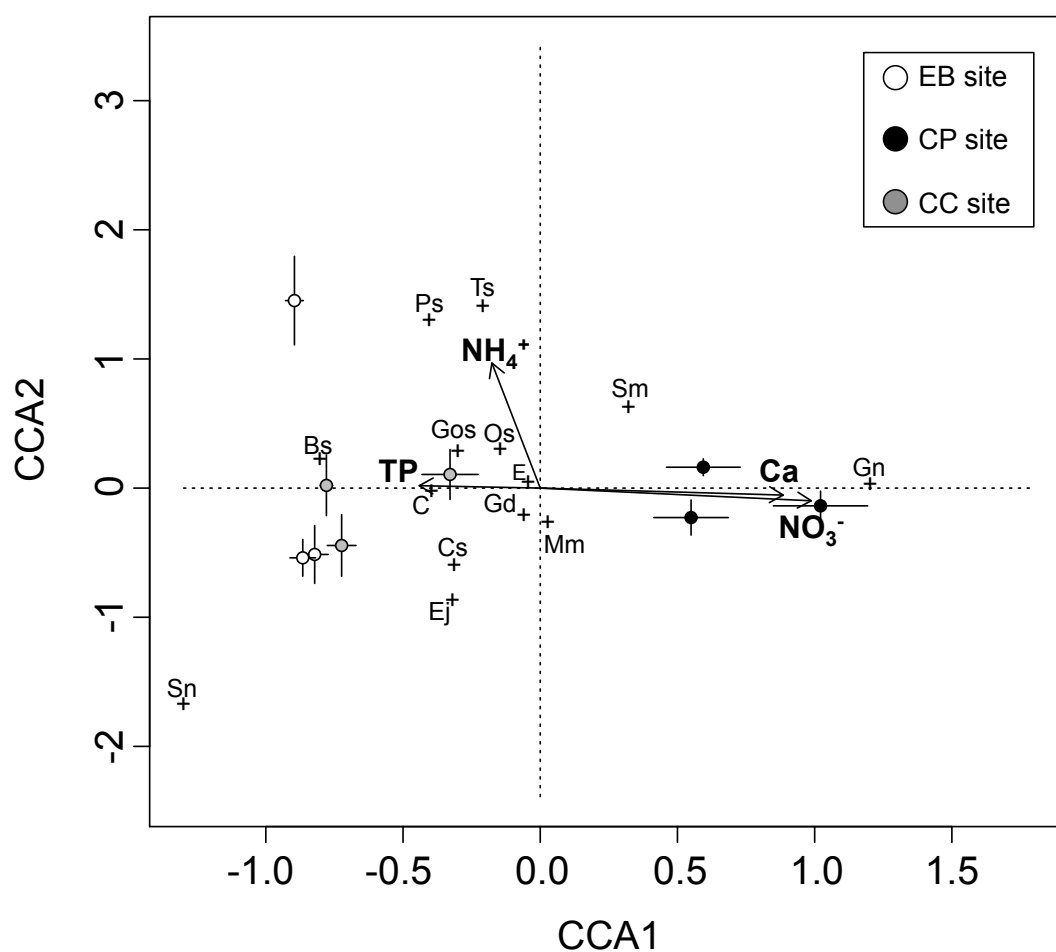


Fig. 3-4 Canonical correspondence analysis (CCA) ordination of stream invertebrate community composition in nine streams. Explanatory variables selected by forward selection are shown as arrows: Ca, calcium concentration and TP, total phosphorus. White, black and grey circles indicate site scores (mean $\pm$ 1 SE) of evergreen broad-leaved forests (EB), cedar plantations (CP) and clear-cut cedar plantations (CC), respectively. Invertebrate taxa are abbreviated by symbols (+) labels: Gn, *Gammarus nipponensis*; Gd, *Geothelphusa dehaani*; Cs, *Cinygmula* sp.; Ej, *Ephemera japonica*; Bs, *Baetis* sp.; Ps, *Paraleptophlebia* sp.; Sm, *Stenopsyche marmorata*; Gos, *Goerodes* sp.; Os, *Oyamia* sp.; Sn, *Sweltsa nikkoensi*; C, Chironomidae; Ts, *Tipula* sp.; Mm, *Mataeopsephus maculatus*; E, Elmidae.

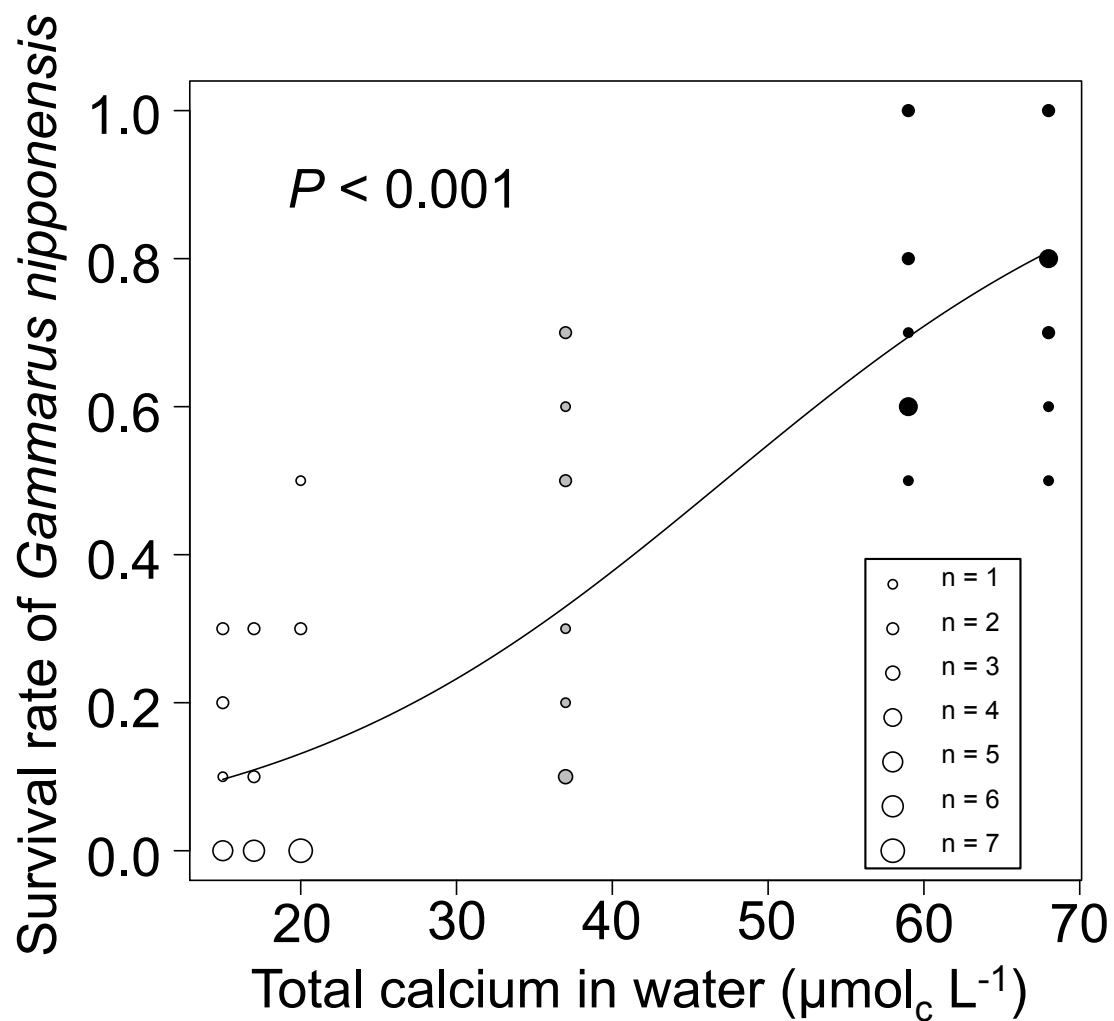


Fig. 3-5 Relationship between calcium concentration in stream water and survival rate of *Gammarus nipponensis* during the field experiment. White, black and grey circles indicate evergreen broad-leaved forests (EB), cedar plantations (CP) and clear-cut cedar plantations (CC), respectively. The size of circles represents the number of experimental cages (10 cages were placed in each site). The regression curve of a logistic model is shown.

Chapter 4

**Light intensity alters effects of nitrogen enrichment on litter decomposition and
invertebrate colonization in a stream ecosystem**

INTRODUCTION

Nutrient mobilization is one of the most important factors affecting terrestrial and aquatic systems world-wide that has many consequences for both communities and ecosystem processes (Smith et al. 1999, Robinson & Gessner 2000, Matson et al. 2002). In aquatic ecosystems such as streams, nitrogen loading is increasing due to human activities (Galloway & Cowling 2002; Elliott et al. 2007, Elser et al. 2009). Because of this situation, to estimate the consequences of nitrogen load, experimental nutrient additions in stream ecosystems have been conducted throughout the world (e.g. Cross et al. 2006, Davis et al. 2010a, 2010b Connolly & Pearson 2013). Increase in nutrient availability enhance the quality of leaf litter for stream macroinvertebrates (Suberkropp & Wallace 1992, Graça et al. 2001, Jabiol & Chauvet 2012). And then the increase in nutrient availability in stream ecosystem cause changes in litter decomposition rate and macroinvertebrate community composition (Elwood et al. 1981, Gulis & Suberkropp 2003, Gulis et al. 2004, Benstead et al. 2005, Greenwood et al. 2007). However previous studies have less noticed another environment factors that might modify the effects of nutrient enrichment on litter decomposition and macroinvertebrates.

Light availability might increase periphyton biomass on leaf litters. Because nutrient concentration in periphyton is usually higher than in leaf litter (Sterner & Elser 2002), the periphyton on the litters might enhance the quality of leaf litter for macroinvertebrates. And light intensity influences the development and biomass of biofilms (Ledger & Hildrew 1998) whose quality as food is likely to be greater when algae are present (Lamberti 1996; Huggins et al. 2004). Furthermore, increase in

periphyton with high quality might affect colonization of invertebrates because stream invertebrates have feeding plasticity (Friberg & Jacobsen 1994). Therefore, litter decomposition rate and colonization of invertebrate might be affected by not only nutrient enrichment but also light intensity in stream. In fact, Franken et al. (2005) demonstrated the positive effect of light intensity on litter decomposition associated microbe and shredders, and the growth of shredder increased with light intensity in a laboratory experiment. They suggested that the results were caused by enhancing the productivity of the periphyton that might increase quality of the leaf litters (Burrell & Ledger 2003).

Because biomass and quality of periphyton are generally influenced by nutrient and light availability (Fanta et al. 2010, Kohler et al. 2012), increase in the two might produce synergistic effects on the litter decomposition and stream invertebrate colonization. However, there is no study manipulated both light and nutrient and verified effect on litter decomposition and invertebrate colonization in stream ecosystem. Then we have to conduct an experiment in various light and nutrient availability, to identify the effects of light intensity on litter decomposition and invertebrate community. We predicted that effects of nitrogen enrichment on litter decomposition rate and macroinvertebrates community would be altered by changes in light intensity.

METHODS

Study site

This study was conducted from October to December 2009 in the Horonai Stream (14 km long) running through the Tomakomai Experimental Forest of Hokkaido University (TOEF; 42°43'N, 141°36'E), south-western Hokkaido, Japan. This stream originates from a spring and its bed is underlain by fine pumice, and have very low nutrient levels are present in the stream water (Ohta et al. 2011). The middle reach of the stream, about 5 km upstream from the river mouth, was chosen as the study site, in which riparian forest were sparse and the canopies rarely covered surface of the stream. We selected a 80 m stretch as the study reach in the site, and created four treatment plots (5 × 15m), in which light intensity, current velocity and water depth were relatively uniform (light intensity in a cloudy day: $186.4 \pm 41.8 \mu\text{mol m}^{-2} \text{s}^{-1}$, current velocity: $24 \pm 2 \text{ m s}^{-1}$, water depth: $20 \pm 5 \text{ cm}$). We selected the two plots at downstream side as fertilization plots, and the two plots at upstream side as unfertilization plots. And then we covered one half of the treatment plots with shade curtain (Shinsei, B0088VF5MG, Fukushima, Japan) that light intensity can be reduced by 90 percent (Fig. 4-1). We named the fertilized and no covered plot as FL (fertilization and light) treatment, the fertilized and covered plot as FD (fertilization and dark) treatment, the unfertilized and no covered plot as L (light) treatment and the unfertilized and covered plot as D (Dark) treatment.

Field experiment

We made a nutrient injection system at central part of the study reach. We placed large water storage tank (2000 L) on the riverside, and charged stream water with ammonium nitrate (NH_4NO_3). We connected a 10-meter hose that transected the stream to the tank,

and continuously dripped water solution of NH_4NO_3 into the stream from 20 October 2009 to 6 December 2009 (~ 48 d) (Fig. 4-1). Nitrogen concentrations in the fertilized treatments plots were actually increased during the experiment and the variations between plots and within a plot were very few ($\text{DIN}, 153.4 \pm 24.8 \mu\text{gL}^{-1}$), whereas in the unfertilized treatment plots the concentrations during the same time period were comparable to the pretreatment period ($\text{DIN}, 23.4 \pm 4.1 \mu\text{gL}^{-1}$). The nitrogen in the stream decreased in concentration toward the lower reaches and was similar level as unfertilized treatment plots about 100 m from the injection points

Oak (*Quercus crispula*) leaves were collected at abscission in 2009 using litter traps and dried at 60 °C for 72 h in a drying oven. These dried leaves (10 g) were placed in 20×20-cm nylon bags with mesh size of 5 mm (coarse mesh bags) and 0.2 mm (fine mesh bags). The two mesh sizes were used to include (5 mm) or exclude (0.2 mm) the access of invertebrates. We deployed the 200 litter bags in total containing oak leaves in the treatment plots at 20 October to determine breakdown rates, and also placed unglazed ceramic tiles (10 × 10cm) on each side of the nylon bags to check the periphyton biomass among the treatments.

Five replicate bags of each mesh size and the ceramic tiles on each side of them were removed randomly after 4, 8, 16, 32 and 48 days of incubation, placed in zip-lock bags and transported in cool box to the laboratory. Within 12 h of collection, the invertebrates in the 5-mm mesh bags were removed from litter bags and preserved in 99 % ethanol. Periphyton samples were collected by brushing the surface of the ceramic tiles and rinsing with distilled water. These suspensions were filtered onto glass

filters (Whatman No. 1822, Maidstone, U.K.), and stored in a freezer at -30 °C until analysis.

Sample processing

The remanding leaf litter and sediment particles in coarse mesh bags were dried at 60 °C for 72 h in a drying oven, and ashed at 500 °C for 3 h in an electric muffle furnace (KM-420; Advantec, Tokyo, Japan) and ash-free dry mass (AFDM) was calculated as the difference in mass before and after ashing to estimate decomposition rate and fine particulate organic matter (FPOM). The invertebrate samples were identified to the lowest possible taxonomic level, usually genus or species.

Chlorophyll *a* (mg m^{-2}) was used as a measure of periphyton biomass. We placed filtered periphyton samples in 90% acetone at 5°C for 24 h to extract the pigments. Pigments in the solution were measured using a spectrophotometer (Shimadzu, UV-3150, Kyoto, Japan). The data were converted to chlorophyll *a* estimates following the procedures outlined in UNESCO (1966).

We measured the chemical properties of the leaf litters of each sampling day. Carbon and nitrogen concentrations of the leaf litters and detritivores were determined using a C/N analyzer (Sumigraph NC-900, Sumika Chemical Analysis Service, Osaka, Japan). To measure the concentration of phosphorus, samples of leaf-litters and detritivores were ashed at 490°C for 2 h, weighed and extracted with 15-mL 1 M HCl at 80°C for 1 h. The concentration of phosphorus in the extraction liquid was determined

using an inductively coupled plasma (ICP) atomic emission spectrometer (ICPE-9000, Shimadzu, Kyoto, Japan).

Statistical Analysis

Decomposition rates were estimated by linear regression of transformed data (negative exponential model $M_t = M_0 \cdot e^{-kt}$, where M_0 is the initial mass of the litters, M_t is the remaining mass at time t and k is the decomposition rate). Differences in k were determined with analysis of covariance (ANCOVA) followed by Tukey's test to compare slopes among treatments. Periphyton biomass, AFDM remaining in fine and coarse mesh bags, C, N and P concentration and FPOM were analysed using one-way analysis of variance (ANOVA) at each sampling days with treatment as an independent variable, followed by post hoc comparisons using Tukey's test.

Relationships between abundance of invertebrates and concentration of nutrients in litters, FPOM in each litter bag, light intensity and periphyton biomass were examined using stepwise generalised linear model (GLM). We selected the best GLM by downward stepwise selection according to the Akaike Information Criterion (AIC). We used the likelihood ratio test to determine whether the data supported selected models over a null model.

We performed redundancy analysis (RDA) to explore the relationships between species composition and physiochemical properties of stream (e.g. nutrient concentration and light intensity). Before conducting RDA ordination, we selected the most important explanatory variables from all physical and chemical properties of

stream water by forward stepwise selection based on AIC and Monte Carlo permutation tests.

All statistical analyses were conducted with R Version 2.9.2. software.

RESULTS

Leaf litters and periphyton

Periphyton biomass on the tiles similarly changed among treatments except for D treatment (Fig. 4-2b). FPOM in the litter bags increased with time and were significantly higher in FL treatment than the others at 48 days (Tukey-HSD, $P < 0.001$, Fig. 4-2a). N concentration of leaf litters increased with time and were significantly higher in FL than FD treatments than in L and D treatments at 48 days (Tukey-HSD, $P < 0.05$, Fig. 4-3b). P concentration of leaf litters increased with time and were significantly higher in FL treatment than the others at 48 days (Tukey-HSD, $P < 0.001$, Fig. 4-3c).

Litter decomposition

The decomposition in fine mesh bag is regarded as microbial decomposition. The microbial decomposition rates, k , did not differ significantly among treatments (ANCOVA, $P > 0.05$). However, AFDM remaining in fine mesh bags differed significantly among treatments at 32 (one-way ANOVA; $F_{3,16} = 8.01$, $P = 0.002$) and 48 (one-way ANOVA; $F_{3,16} = 14.69$, $P < 0.001$) days (Fig. 4-4a). AFDM remaining in the bags were significantly lower in fertilized treatments plots at 32 and 48 days

(Tukey-HSD, $P < 0.05$, Fig. 4-4a).

The decomposition in coarse mesh bag is regarded as decomposition associated with microbe and invertebrates. The decomposition rates in the bags, k , did not differ significantly among treatments (ANCOVA, $P > 0.05$). However, AFDM remaining differ significantly among treatments at 32 (one-way ANOVA; $F_{3,16} = 6.31$, $P = 0.005$) and 48 (one-way ANOVA; $F_{3,16} = 9.90$, $P < 0.001$) days (Fig. 4-4b). AFDM remaining in coarse mesh bags were significantly lower in FL treatment at 32 and 48 days (Tukey test, $P < 0.05$, Fig. 4-4b).

Invertebrate assemblage

Jesogammarus yesoensis, *Choroterpes* sp., *Isoperla* sp. and *Gyraulus chinensis* accounts for 93.9 % of the sampled invertebrates. Abundances of *J. yesoensis* that is a leaf litter feeder (shredder) were significantly higher in FL and L treatments than the others at 32 days (Fig. 4-5a). Abundance of *G. chinensis* that is a grazer increased sharply after 8 days in FL treatment (Fig. 4-5b). Abundances of *Isoperla* sp. and *Choroterpes* sp. that are FPOM feeders (collector-gatherer) were significantly higher in FL treatment than the others at 48 days (Fig. 4-5c, d). The model about abundance of dominant invertebrates that had lowest AIC value at each sampling days included FPOM and concentration of nutrients (Table 4-1).

Forward selection revealed that most of physiochemical properties significantly explained the variation in species assemblages across these four treatments. All, first, and second axes significantly explained, 24.69, 17.51 and 4.25 % respectively,

of the species assemblage variation (Monte Carlo permutation test: $P < 0.05$). The RDA ordination showed that the community structure of stream invertebrates at each sampling day clearly changed in FL treatment along with the first axis, which was correlated with P and N concentrations in litters and FPOM (Fig. 4-6). *Isoperla* sp. and *Choroterpes* sp. had a large score on the first axis, and its high abundance characterized the FL treatment at 48 days. *G. chinensis* that was high abundance in FL treatment correlated with light intensity, which were high level in FL treatment.

DISCUSSION

This is the first study showing the effects of nitrogen enrichment may be altered by light intensities. The light intensity is easily changed by the density of riparian forest or water turbidity. We should regard the light intensity on the streambed when we consider the effect of nitrogen enrichment.

Litter decomposition

AFDM remaining at day 32 and 48 days were significantly lower in FL treatment than the others only in coarse mesh bags (Fig. 4-4). However, abundances of *J. yesoensis* that is a shredder were significantly higher not only FL treatment but also L treatment at day 32 (Fig. 4-5). We take particular note of what *G. chinensis* dominated greatly in the FL treatment (Fig. 4-5). Because freshwater snails powerfully scrape the biofilm on the substrate (Schaller et al. 2011), leaf litters in our site might be scraped by them and accelerated the breakdown. Previous studies manipulated either light (Franken et al.

2005) or nutrient (Meyer & Johnson 1983; Gulis & Suberkropp 2003, Gulis et al. 2006), and verified the effects on litter decomposition. Our results newly discovered synergy between light intensity and nutrient enrichment.

On the other hand, AFDM remaining in coarse mesh bags (decomposition associated with microbes and invertebrates) did not differ between FD treatment and unfertilized treatments during the experiment. This result conflict with previous studies that conducted nutrients enrichment in dark forest streams (Gulis & Suberkropp 2003, Gulis et al. 2006; Greenwood et al. 2007). The reason might come from the fact that the increasing amount of nutrient concentration in our stream was relatively lower than above studies. Furthermore, because low order stream is frequently deficient in phosphorus (Horne & Goldman, 1994), the conflict between previous studies and our results mainly coursed by non-addition of phosphorus. Ferreira et al. (2006) showed nitrogen addition affected microbial decomposition of litter but not decomposition by invertebrates in a forested stream. Our results showed AFDM remaining in fine mesh bags (microbial decomposition) at 32 and 48 days were significantly higher in FD and FL treatments, but not in coarse mesh bags. Therefore, when we considered the effects of nitrogen enrichment on litter decomposition, light intensity should be taken into account.

Invertebrate colonization in the litter bags

P concentration in litters and FPOM in the litter bags that may affect invertebrate colonization were significantly higher in FL treatment (Fig. 4-2, 3). Because periphyton

exudates are used as an energy source of microbe containing many nutrient (Ledger & Hildrew 1998; Burrell & Ledger 2003), light availability (i.e. periphyton productivity) might have a positive effect on nutrient concentration in the litters. This means synergy effects on change in quality and processing of leaf litters might be expected when light and nutrient availability are increased. In consequences, P concentration in litters might be increased only in FL treatment at 48 days. Periphyton productivity might also be increased by synergy effects of light and nutrient availability. In fact, periphyton biomass in FL treatment were similar to other treatments during experimental period despite high grazing pressure by *G. chinensis*. Therefore, significant increase in the abundance of *G. chinensis* might be produced by high productivity of periphyton in FL treatment. And significant increase in the abundances of *Isoperla* sp. and *Choroterpes* sp. that are collector-gatherer might be produced by increasing FPOM in the litter bags and nutrients concentration of litters in FL treatment at 48 days (Table 4-1, Fig. 4-5). Differences in abundances of *G. yesonensis* among treatments might be produced mainly by light intensity (Table 4-1, Fig. 4-5). Because gammarid amphipod can exhibit positive phototaxis under some conditions (Brown and Thompson 1986), *G. yesonensis* might assemble in FL and L treatments. N and P concentrations in litters and FPOM in the litter bags greatly changed in FL treatment. The results of RDA that showed change in the community at FL treatment mainly affected by N and P concentrations in litters and FPOM.

Nutrient loading is a major threat to stream ecosystem worldwide, leading to change in biophysical processes (Woodward et al. 2012). However, our results showed

effects of nitrogen enrichment on litter decomposition and invertebrate colonization could be altered by another environmental factors, such as light availability that easily changed by the density of canopy cover due to riparian forest dynamics. This means we have to conduct the experiment under many conditions of light and nutrient to estimate the relative strengths and relationships between the two.

Table 4-1 Relationships between abundance of each dominant invertebrate and each variable.

Species name	Sampling days	Best fit model	Coefficient	P
<i>Jesogammarus yesoensis</i>	4	C + light	4.874 ± 0.530	**
	8	FPOM + N + P + periphyton + light	4.694 ± 0.348	**
	16	FPOM + P + peri + light	2.976 ± 0.182	**
	32	C + N + P + peri + light	3.793 ± 0.571	**
	48	FPOM + C + N + peri + light	4.235 ± 0.518	**
<i>Isoperla</i> sp.	4	C + N	-2.346 ± 1.395	n.s.
	8	FPOM + peri	0.861 ± 0.334	**
	16	C + N + peri + light	-2.587 ± 0.878	**
	32	FPOM + C + N + P + peri	6.710 ± 0.715	**
	48	C + N + P + peri + light	2.013 ± 0.312	**
<i>Choroterpes</i> sp.	4	C + N + P + peri + light	-7.676 ± 1.985	**
	8	FPOM + C + P + peri + light	7.85 ± 1.779	**
	16	C + P + peri	0.962 ± 0.945	**
	32	FPOM + P + peri + light	4.846 ± 0.514	**
	48	FPOM + N + P + peri + light	1.853 ± 0.257	**
<i>Gyraululus chinensis</i>	4	FPOM + C + N + P + peri + light	-3.834 ± 0.893	**
	8	FPOM + C + N + P + peri	-0.807 ± 0.465	**
	16	FPOM + C + P + peri	1.505 ± 0.367	**
	32	FPOM + C + N + P + light	6.872 ± 0.401	**
	48	FPOM + N + P + peri + light	-0.473 ± 0.222	**

** $P < 0.001$

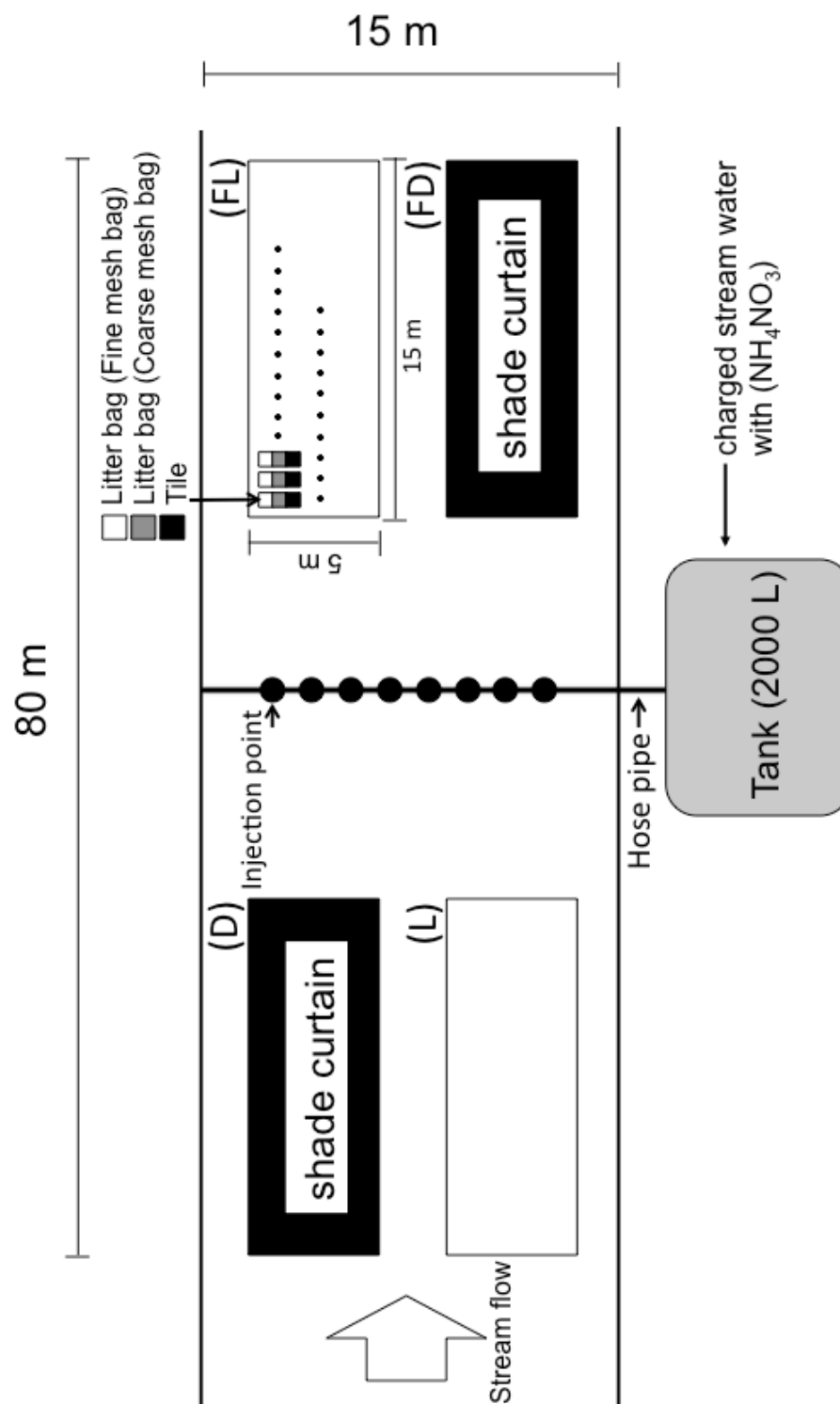


Fig. 4-1 Schematic diagram of our site. Balck and white oblong bar indicate each treatment plots.

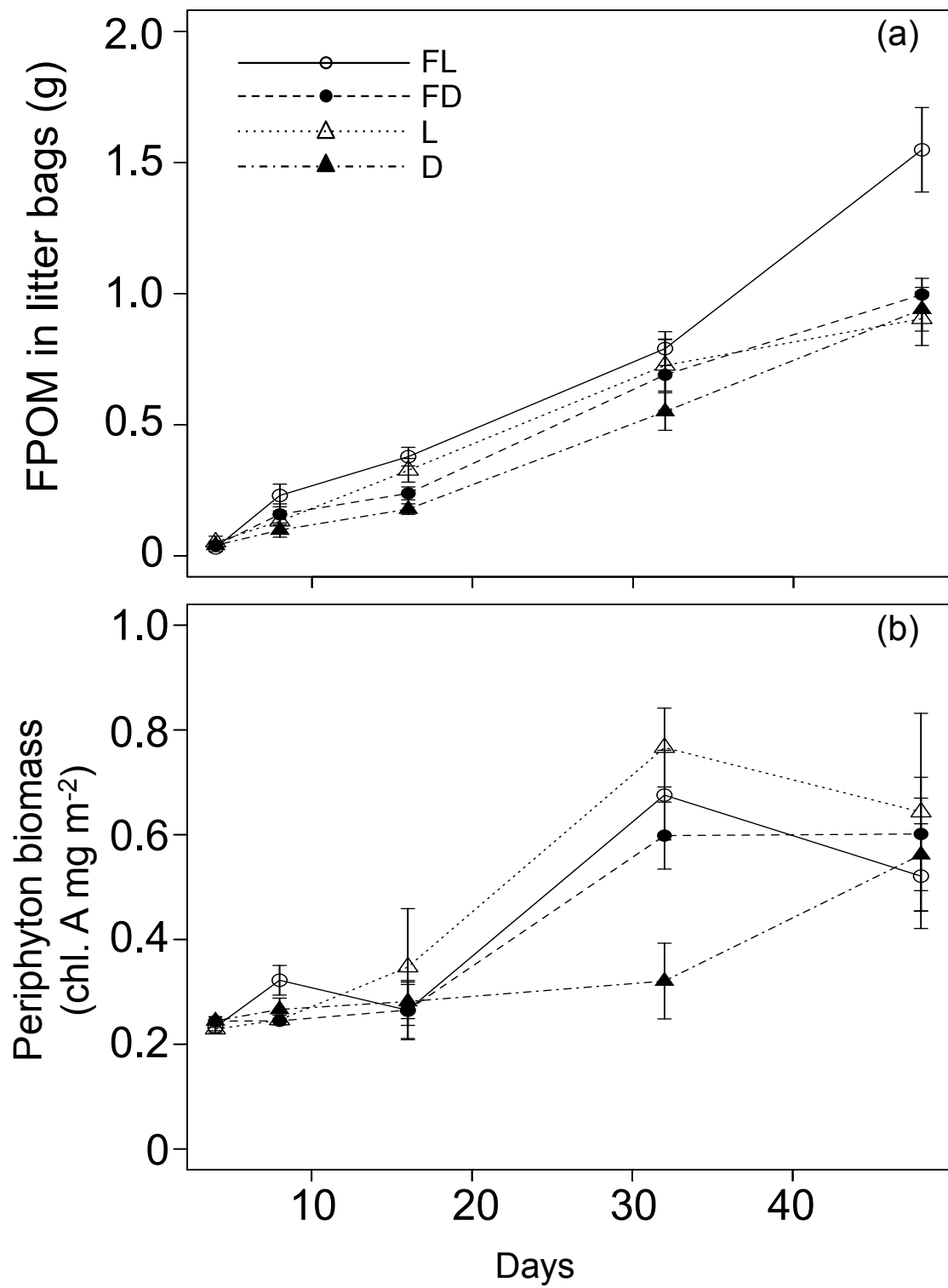


Fig. 4-2 Fungal biomass (a), FPOM in the cause mesh bags in complete time series stream. Values are mean $\pm$ SE.

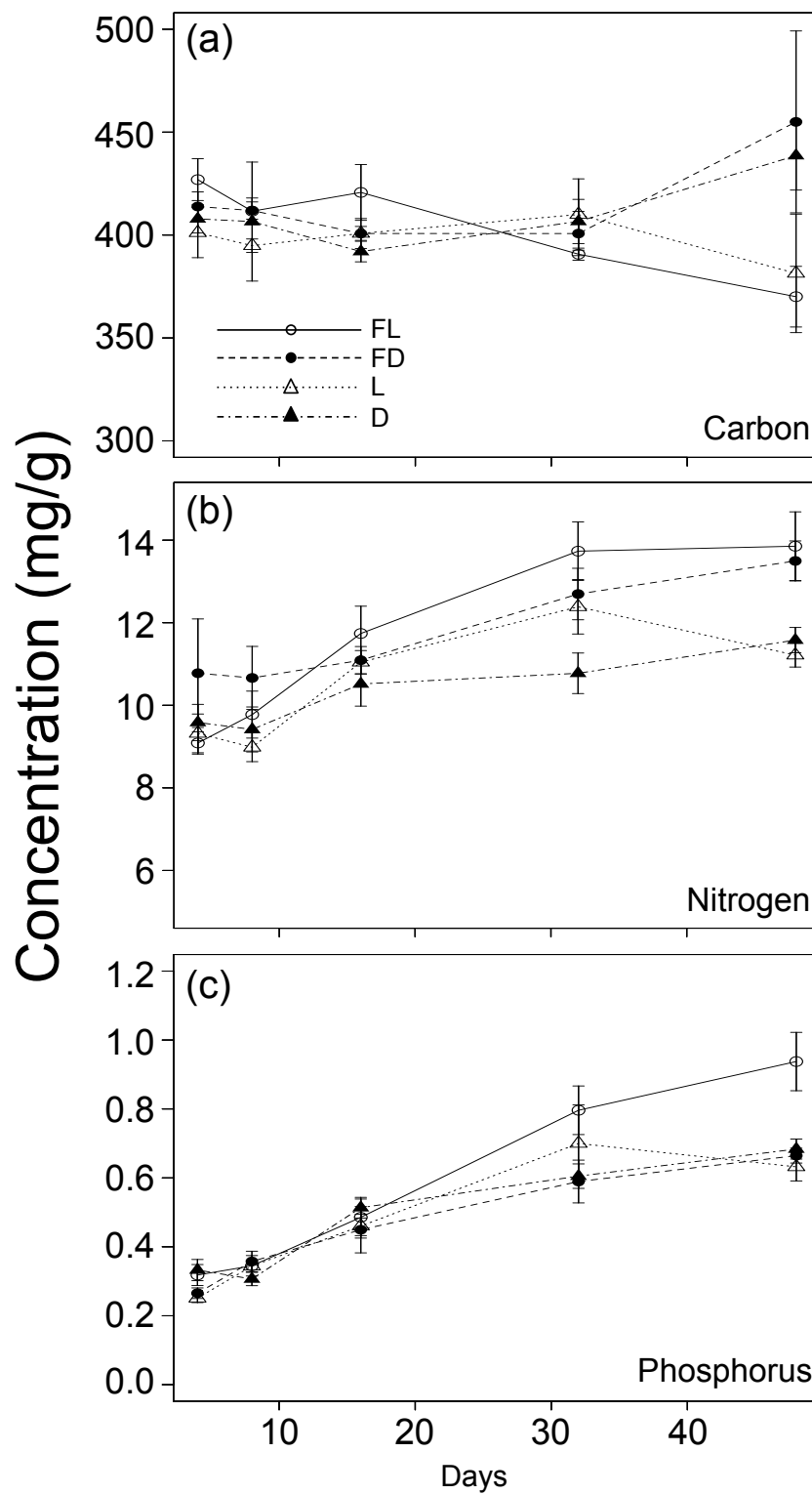


Fig. 4-3 Carbon (a), nitrogen (b) and phosphorus (c) concentration of leaf litters in coarse mesh bags in complete time series stream. Values are mean $\pm$ SE.

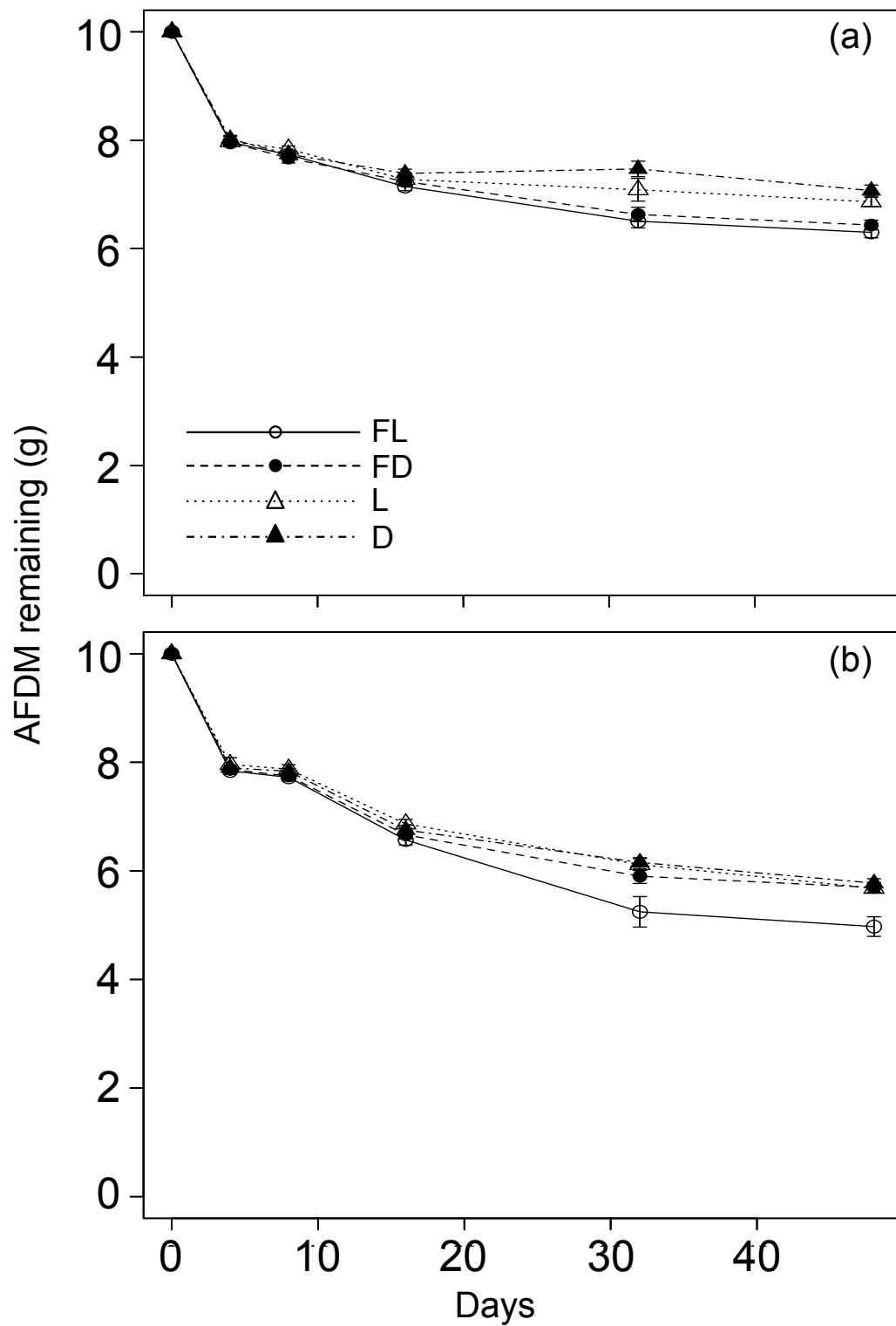


Fig. 4-4 Decomposition of leaf litters in fine (a) and coarse (b) mesh bags in complete time series stream. Values are mean $\pm$ SE.

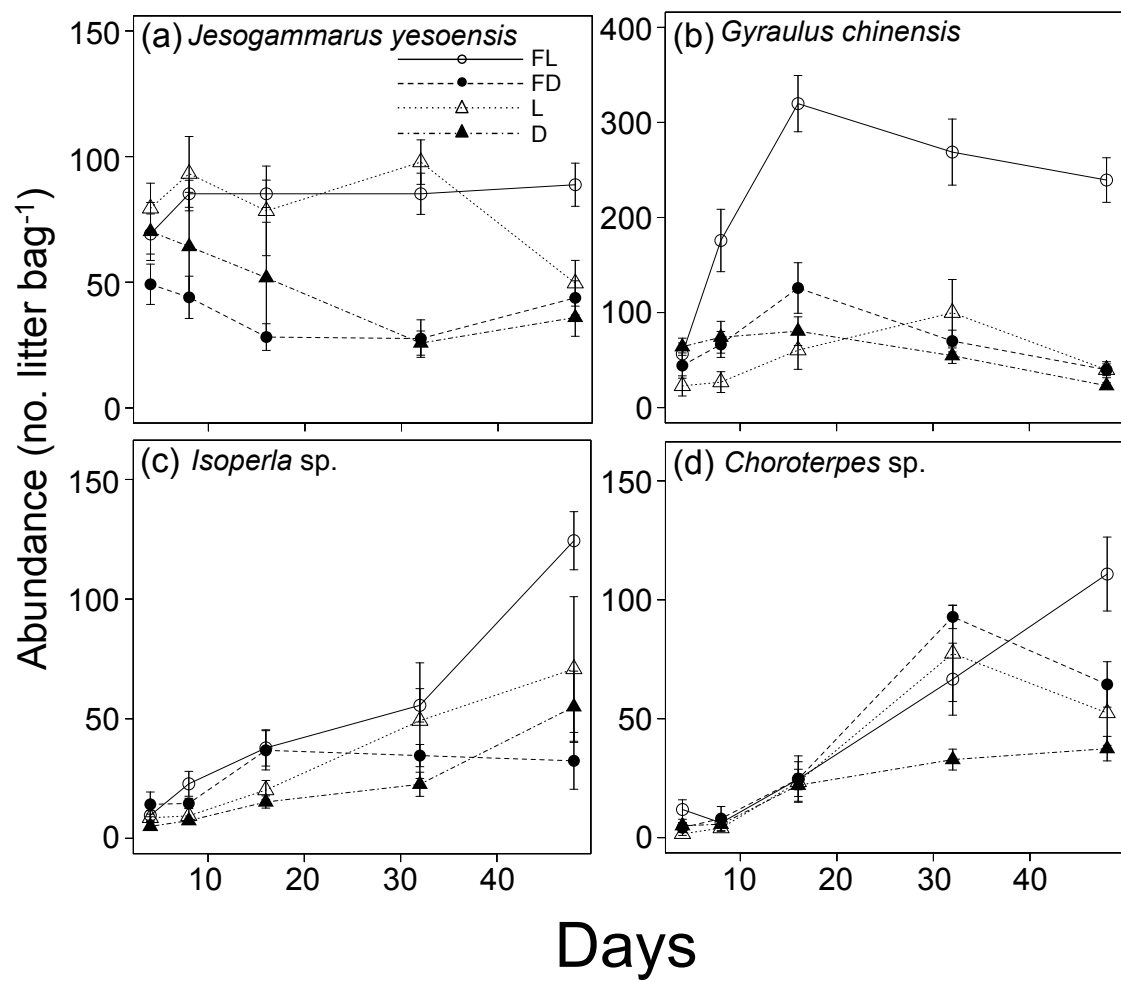


Fig. 4-5 Effect of colonization time on the absolute abundance of four dominant taxa in the coarse mesh bags in complete time series stream. Values are mean $\pm$ SE.

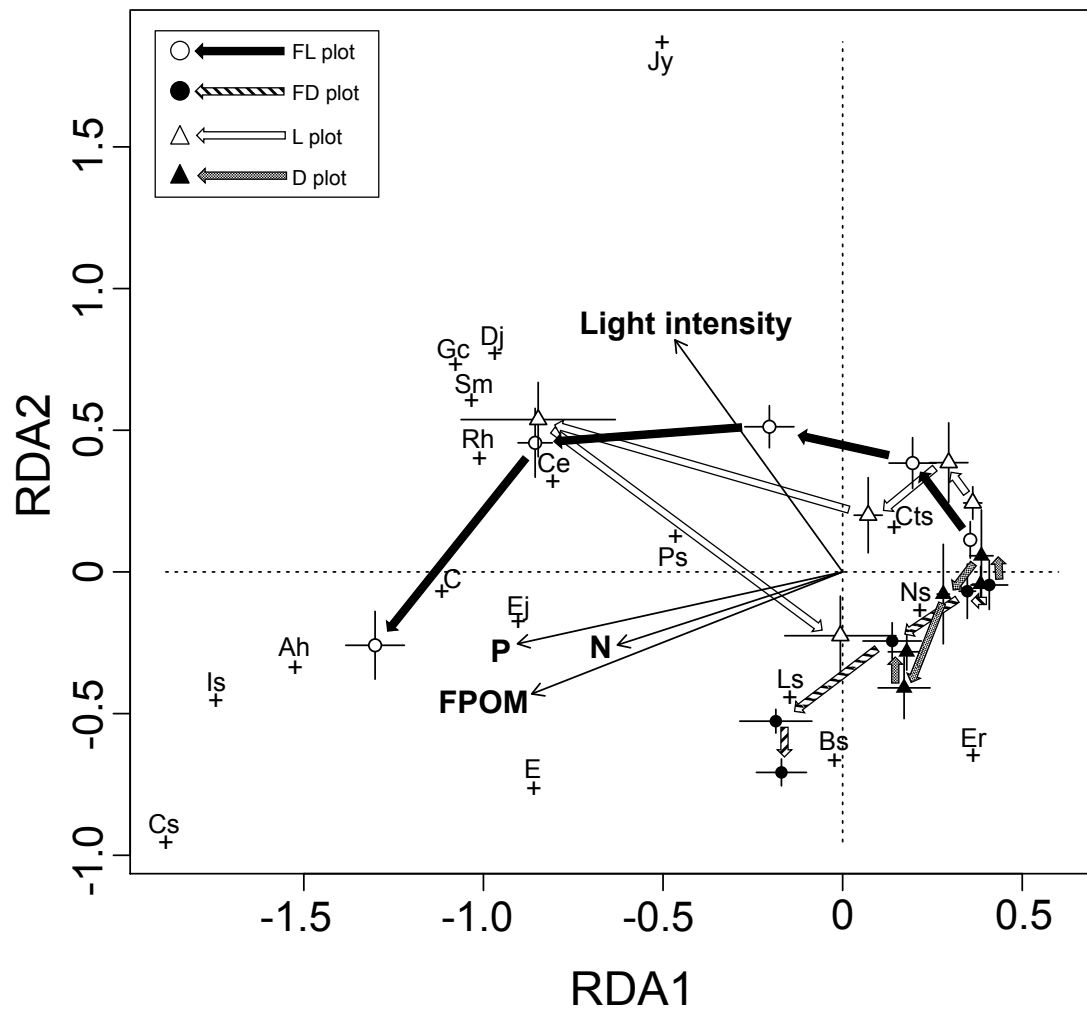


Fig. 4-6 Redundancy Analysis (RDA) ordination of stream invertebrate community composition in four treatments each sampling day. Explanatory variables selected by forward selection are shown as arrows: FPOM, fine particulate organic matter, P, phosphorus concentration and N, nitrogen concentration in the litters. Black-and-white circles and triangles indicate treatment scores (mean $\pm$ SE) respectively. The time series variations of each treatment score indicate the arrows connecting between symbols. Invertebrate taxa are abbreviated by symbols (+) labels: Jy, *Jesogammarus yesoensis*; Is, *Isoptera* sp.; Cs, *Choroterpes* sp.; Gc, *Gyraulus chinensis*; Ns, *Nemoura* sp.; Ce,

Cincticostella elongatula; Bs, *Baetis* sp.; Ej, *Ephemera japonica*; Ls, *Lepidostoma* sp.;
Dj, *Dicosmoecus jozankeanus*; Er, *Eubasilissa regina*; Sm, *Stenopsyche marmorata*; Ah,
Asellus hilgendorfi; Rh, *Rhyacophila hokkaidensis*; Ps, *Pisidium* sp.; C, Chironomidae;
Cts, *Ctenacroscelis* sp.; E, Elmidae.

Chapter 5

Stoichiometry meets diversity effects on decomposition in the freshwater ecosystem

INTRODUCTION

Rapid losses of biodiversity are occurring on a global scale due to human impacts on ecosystems (Sala et al. 2000, Dudgeon et al. 2006), and understanding the consequences of biodiversity loss to ecosystem functioning is an urgent issue. Many studies in the last two decades have revealed relationships between biodiversity and ecosystem functioning (B-EF) (Tilman et al. 1996; Balvanera et al. 2006; Riess et al. 2009; Hooper et al. 2012). For example, the litter decomposition rate increases with the diversity of the detritivore assemblage in freshwater (Jonsson & Malmqvist 2000; McKie et al. 2008). However, the mechanisms underlying these observed biodiversity effects on decomposition processes are not well understood (Giller et al. 2004; Gessner et al. 2010). In fact, some studies have shown that greater species richness is associated with faster decomposition (Jonsson & Malmqvist 2000; McKie et al. 2008), while others have shown neutral outcomes; negative effects have also been reported (McKie et al. 2008, 2009). Many B-EF studies have focused on linking empirical observations with concepts such as the complementarity or facilitation effects (e.g. Cardinale et al. 2002; Cardinale et al. 2007; Riess et al. 2011). Complementarity effects on litter decomposition rates are driven by functional dissimilarity in traits such as body size, feeding efficiency and dietary flexibility among detritivorous species (Heemsbergen et al. 2004; Gessner et al. 2010; Riess et al. 2011). However, relationships between diversity and decomposition cannot be explained only with above factors (Gessner et al. 2010).

Basal resources in food webs vary widely in their elemental compositions and

resource qualities (Cross et al., 2005), whereas consumers often operate within more tightly constrained limits (Sterner & Elser, 2002). This often gives large stoichiometric imbalances between consumers and resources that are likely to have serious consequences for the growth and reproduction of consumers in streams (Sterner & Elser 2002; Ohta et al. 2011). Furthermore, recent studies have revealed that material ratios of detritivores, such as the C (carbon): N (nitrate): P (phosphorus) ratio, vary widely among species in stream ecosystems (Evans-White et al. 2005; Persson et al. 2010). Therefore, the strength of the limitation effect might depend on the C: N: P ratio in the body of a consumer. Therefore, feeding behaviors may vary with the C: N: P ratio in the body. We addressed stoichiometric divergence among detritivores as the functional dissimilarity that affects litter decomposition.

The C: nutrient ratios of leaf litters vary widely among species, and these stoichiometric differences might affect litter decomposition by detritivores (Woodward 2009; Manzoni et al. 2010). Zimmer et al. (2005) suggested that complementarity effects on decomposition mediated by detritivores vary with resource quality. Litter assemblages usually contain various species of leaf litters, with nutrient qualities varying among species. The litter of *Alnus*, which can be symbiotic with a nitrogen-fixing bacterium, has high nitrogen content, while the litters of other species, such as *Ulmus glabra* and *Pterostyrax hispida*, contain relatively high amounts of phosphorus (Osono & Takeda 2004; Schindler & Gessner 2009). These differing nutrient qualities of leaf litters among species might affect complementarity effects on decomposition.

In aquatic ecosystems, differences in C: N: P ratios affect fungal biomass on leaf litter (Jabiol & Chauvet 2012). Aquatic hyphomycetes fungi enhance litter quality (e.g. hardness of litter, nutrient concentration in litter) to macroinvertebrate shredders, thereby indirectly facilitating decomposition (Suberkropp, 1992; Graça, 2001; Jabiol & Chauvet 2012). Therefore, because of its original C: N: P ratio and fungal colonization, the quality of leaf litter deposited on a streambed might differ widely among species of litter, and might affect the feeding behavior of detritivores.

Various laboratory studies have manipulated either plant or detritivore diversity (e.g. Jonsson & Malmqvist 2000; Mckie et al. 2008, 2009; Jabiol & Chauvet 2012). However, to verify the effects of stoichiometric differences in detritivores and leaf litters on decomposition rates, we must manipulate not only plant litter diversity but also detritivore diversity simultaneously.

We addressed the effect of stoichiometric differences among detritivores and its diversity on the decomposition rate of litter mixtures, and conducted microcosm experiments. We predicted that (1) detritivores with nutrient-rich bodies might tend to consume litters with low C: nutrient ratios, while detritivores with nutrient-poor bodies might tend to consume litters uniformly; and (2) stoichiometric differences among detritivores and their diversity affect litter decomposition rate.

METHODS

We manipulated the stoichiometric diversity of stream detritivores, and placed them into microcosms with leaf litter mixtures. Thirty-eight days after the initiation of the

experiment, we calculated the litter decomposition rate per microcosm, and compared this among treatments.

Invertebrates and field sampling

The detritivores (*Jesogammarus yesoensis*, *Sternomoera yezoensis*, *Goerodes satoi*, *Nemoura* sp., *Amphinemura* sp. and *Cincticostella nigra*) were collected from the upper and middle reaches of Horonai Stream, which runs through the Tomakomai Experimental Forest of Hokkaido University, southwestern Hokkaido, Japan (TOEF: 42°43'N, 141°36'E). This stream originates from a spring, and its bed is underlain by pumice. Immediately prior to the experiment, the C, N and P concentrations in the bodies of 12 randomly selected individuals of each species were measured, as described below. We then classified *J. jesoensis*, *S. yezoensis*, *G. satoi* as species with nutrient-rich bodies (RB), and *Nemoura* sp., *Amphinemura* sp. and *C. nigra* as species with nutrient-poor bodies (PB) (Table 5-1). *J. jesoensis*, *S. yezoensis*, *G. satoi*, *Nemoura* sp. and *Amphinemura* sp. were classified as shredders that chew the leaf litter, and *C. nigra* was classified as a collector-gatherer that feeds on fine detritus (Takekado 1995) (Table 5-1). These species are dominant species in the upper reaches of the stream, and feed on the litter deposited on the streambed (Merritt et al. 2008).

Leaf-litters of *Quercus crispula*, *Carpinus cordata*, *Alnus japonica* and *Styrax obassia* were collected in TOEF using litter fall traps made of large nylon nets, just before the beginning of the experiment in late October 2012. The four species have markedly different leaf-litter nutrient qualities (Table 5-2). The collected leaf-litters

were sorted and dried at 60°C for 72 h. Four grams of dried leaf-litter were placed in large litter bags (ca. 20 × 20 cm, 5-mm mesh size), and one gram of dried leaf-litter was placed in small litter bags (ca. 5 × 10 cm, 5-mm mesh size). The placed leaf-litters were chopped into small pieces, to attenuate for the influence of differences in thickness between leaf-litters. We constructed 20 large and 760 small litter bags for each plant litter species, giving 80 large and 3,040 small litterbags in total.

Experimental system

The experiment was conducted from 27 October to 5 December, 2012. We prepared 840 microcosms (cylindrical polyethylene cups with a diameter of 8 cm and height of 24 cm), into which were poured water. Large litter bags were placed into 80 out of the 840 microcosms, while into the other microcosms were placed four small litter bags of different species. Thus, 80 microcosms contained single species litter (SL) and 760 microcosms contained mixed litter of all four species (ML), with all microcosms containing an equal mass of litter. One week after the addition of the litter bags, 12 detritivores were introduced into each microcosm. Single-species detritivores were placed into 120 of the ML, two kinds of detritivores were placed into 300 of the ML, in all 12 combinations, four kinds of detritivores were placed into a further 300 of the ML in all 12 combinations and six kinds of detritivores were placed into 20 of the ML (hereinafter, detritivore-present microcosms). The remaining 20 ML and all SL contained no detritivores (hereinafter, detritivore-absent microcosms) to estimate microbial decomposition rates. The body lengths or head capsule widths of all

detritivores placed into microcosms were measured from digital photographs using ImageJ (version 1.41; US National Institutes of Health, Bethesda, Maryland). We calculated biomass from the body-length measurements using length–mass regression equations published by Smock (1980), Burgherr & Meyer (1997), Johnston & Cunjak (1999) and Miyasaka et al. (2008). The total biomass of detritivores ranged from 23.61 to 7.52 g in each microcosm. The 840 microcosms were randomly deployed in five experimental channels ($2.5 \times 0.7 \times 0.3$ m). Water was supplied at a constant rate to the channels from the nearby Horonai stream to replicate field water temperatures in the microcosms (Fig. 5-1). Water in Horonai stream contains very low nutrient levels throughout the year (Ohta et al. 2011). During the experimental period, we conducted total water exchange triweekly to avoid oxygen deficiencies. We checked all microcosms every day, and if the detritivores in microcosms were dead, they were replaced immediately with alternative individuals of the same body length. The death rate of each species decreased to below 8%.

The litter bags in detritivore-present microcosms were collected on the final day of the experiment, the remaining leaf-litter in each bag was dried and its mass measured. We assumed the rate of decrease of litter in the bags to be the decomposition rate of the litter, and compared litter decomposition rates among litter species in the ML. The combined decomposition rates of the four species of litter in each ML were calculated as g litter dry mass per metabolic capacity. The metabolic capacity of detritivores correlates allometrically with body mass, as described by Kleiber's

relationship (Kleiber 1932), which we used to calculate the per capita metabolic capacity of each species in each microcosm:

$$\text{per capita metabolic capacity} = [\text{per capita mass (mg)}]^{0.75}$$

The exponent of 0.75 describes a general relationship between metabolism and body size across all organisms, and is a useful compromise when species-specific relationships are unknown (Brown *et al.* 2004). The total detritivore metabolic capacity was quantified for each microcosm by summing the per capita metabolic capacities across all individuals and species.

The litter bags in detritivore-absent microcosms were also collected on the final day of the experiment, freeze-dried and the remaining mass of leaf-litter in each bag was measured. The freeze-dried leaf litters were smashed and the following chemical analysis was conducted to estimate the quantity of fungal biomass and the quality of leaf-litters for detritivores during the experiment.

Treatment of samples

The leaf discs in the detritivore-absent microcosms used for ergosterol determination, as a proxy measure of fungal biomass, were freeze-dried and weighed to approximately 20 mg. The ergosterol in the leaf discs were extracted with a 5 mL of hexane mixed with approximately 50 μ L of dichloromethane by ultra-sonification. 0.3 ml of KOH methanol solution (8 g L⁻¹) was added into the extract. The extract was hydrolyzed for 120 min at

120°C under reflux. After removing excess KOH and hydrolyzed lipids by purified water, the organic solvent phase (hexane) was concentrated using rotary evaporators (N-1110V-WD; EYELA, Tokyo, Japan). The extract was further concentrated with a gentle Argon flow to several 10µL, then, 1µL of the extract was injected into a gas chromatograph connected to a mass spectrometer (GC-MS; Agilent 7890A GC, 5975C MSD Agilent Technologies Inc., Santa Clara CA, USA). Ergosterol in the samples was quantified by comparing the MS response with that of internal standard (Cholesterol-2,3,4-13C) which was added into the litter sample before the extraction. A conversion factor of 5.5-mg ergosterol per gram fungal dry mass (Gessner & Chauvet, 1993) was used to calculate fungal biomass per gram of leaf-litter dry mass.

We measured the pre- and post-experiment chemical properties of the leaf litters and detritivores. Carbon and nitrogen concentrations of the leaf litters and detritivores were determined using a C/N analyzer (Sumigraph NC-900, Sumika Chemical Analysis Service, Osaka, Japan). To measure the concentration of phosphorus, samples of leaf-litters and detritivores were ashed at 490°C for 2 h, weighed and extracted with 15-mL 1 M HCl at 80°C for 1 h. The concentration of phosphorus in the extraction liquid was determined using an inductively coupled plasma (ICP) atomic emission spectrometer (ICPE-9000, Shimadzu, Kyoto, Japan). The concentration of lignin in leaf litter was estimated by gravimetry according to a standardized method using hot sulfuric acid digestion (King & Heath 1967).

Statistical analysis

The fungal biomass, C, N, P and lignin concentration, C: N and C: P ratios of the leaf-litters were assessed by one-way ANOVA with species of leaf litter and its condition (i.e., fresh-litter, litters in ML and SL after experiments) as an independent variable, followed by *post hoc* comparisons using Tukey–HSD tests.

The litter decomposition rate per metabolic capacity of detritivores was analysed using a generalized linear model (GLM) with stoichiometric combination, variation of body size, number of species, number of feeding type and the number of detritivore species as the explanatory variable. We used the likelihood ratio test to determine whether the data supported selected models over a null model. We calculated Akaike information criteria (AIC) across all models. And then we estimated relative importance of each explanatory variable using the Akaike weights (Burnham & Anderson 2002). Akaike weights (W_i) that is defined by the following equation can be used to evaluate the relative contribution of different variables in the set of the models.

$$W_i = \exp\left(-\frac{\Delta_i}{2}\right) / \sum_{r=1}^R \exp\left(-\frac{\Delta_r}{2}\right)$$

Δ_i : the difference in values of AIC between each model i and the best model having the lowest AIC.

Relative importances of each explanatory variable were defined values that summed W_i of all models incorporated each explanatory variable.

To estimate the effect of detritivore diversity on the decomposition rate, we determined whether there were feeding preferences among detritivore species. We

compared the amount of litter decomposition among litter species in microcosms using data from microcosms into which one species of detritivore had been placed. Data were analysed separately by species of detritivores. Decomposition rates were analysed using one-way ANOVA with litter species, followed by *post hoc* comparisons using Tukey–HSD tests. The coefficient of each explanatory variable (i.e., fungal biomass, C, N, P and lignin concentration, C: N ratio and C: P ratio in leaf-litters) for the dependent variable of litter decomposition rate per detritivore metabolic capacity was estimated using GLM. We used a likelihood-ratio test to determine whether the data supported selected models over a null model. We selected best-fit models in a stepwise fashion using Akaike’s information criterion to examine the contribution of each significant explanatory variable for decomposition rate among leaf litters.

Litter decomposition rates per detritivore metabolic capacity were fitted to generalized linear mixed models (GLMMs) with the number of detritivore species as a fixed factor and detritivore combinations as a random factor. Litter decomposition rates per detritivore metabolic capacity were assumed to follow Gaussian distributions. The statistical significance of the effect of the fixed factor in each model was evaluated using a likelihood-ratio test ($\alpha = 0.05$). When the effect of the number of detritivore species was significant, *post hoc* comparisons using likelihood ratio tests were conducted for all six pairs of detritivore species with significance levels adjusted by Bonferroni’s method ($\alpha = 0.05/6$). To estimate the effects of the stoichiometric diversity of detritivores on litter decomposition rate per detritivore metabolic capacity, litter decomposition rates per detritivore metabolic capacity were fitted to generalized linear

mixed models (GLMMs) with stoichiometric combination as a fixed factor and detritivore combination as a random factor. The statistical significance of the effect of the fixed factor in each model was evaluated using a likelihood-ratio test ($\alpha = 0.05$). The statistical significance of the effect of the fixed factor in each model was evaluated using a likelihood-ratio test ($\alpha = 0.05/9$).

All statistical analyses were performed using the software R, version 3.0.1 (R Development Core Team, 2013).

RESULTS

Leaf litter traits

C (one-way ANOVA: $F = 8.72$, $df = 11$, $P < 0.001$), N (one-way ANOVA: $F = 41.99$, $df = 11$, $P < 0.001$), P (one-way ANOVA: $F = 35.57$, $df = 11$, $P < 0.001$) and lignin (one-way ANOVA: $F = 26.75$, $df = 11$, $P < 0.001$) concentration, and C : P (one-way ANOVA: $F = 82.69$, $df = 11$, $P < 0.001$) and C : N (one-way ANOVA: $F = 108.34$, $df = 11$, $P < 0.001$) ratios differed significantly among litter condition for all four species (Table 5-2). Fungal biomass differed significantly among litter conditions (one-way ANOVA: $F = 15.22$, $df = 7$, $P < 0.001$), and was significantly higher in the leaf litters of *A. japonica* and *S. obassia* in ML (Fig. 5-2, Tukey–Kramer tests, $P < 0.001$), with the microbial decomposition rate showing similar results (Fig. 5-2, one-way ANOVA: $F = 15.22$, $df = 7$, $P < 0.001$, Tukey-Kramer tests, $P < 0.001$).

Litter decomposition

The decomposition rates of ML that were placed with single-species of detritivores, including RB, differed significantly among litter species, and were correlated significantly with some traits, such as fungal biomass, P concentration and C : P ratio (Table 5-3, Fig. 5-3). However, the decomposition rates of ML that were placed with single species of detritivores, including PB, did not differ among litter species (Fig. 5-3). N concentration and C: N ratio were not selected as significant explanatory variables.

All microcosms into which detritivores were placed contained ML, and the decomposition rates described below are the sums of the decomposition rates of each species of litter. Our results showed significant effects of the stoichiometric combination, variation of body size, number of detritivore species and number of feeding type on litter decomposition rates; however, there were no significant effects of species combination (Table 5-4, Fig. 5-4, 5-5, 5-6). Furthermore, the contribution of stoichiometric combination on decomposition was the highest among the explanatory variables. The litter decomposition rate differed significantly with detritivore richness, and increased with the number of detritivore species (likelihood-ratio test, $\chi^2 = 43.703$, $df = 1$, $P < 0.001$). Decomposition rates differed significantly depending on stoichiometric combination (likelihood-ratio test, $\chi^2 = 26.823$, $df = 2$, $P < 0.001$, Fig. 5-6). In particular, there were significant differences in litter decomposition rates among stoichiometric combinations in the microcosms containing two species of detritivore (Fig. 5-6). Decomposition rates in the microcosms containing two species of detritivores with both RB and PB were significantly higher than the decomposition rates in microcosms containing two species of detritivores including only RB (likelihood

ratio test, $\chi^2 = 18.759$, $df = 1$, $P < 0.001$) and microcosms containing two species of detritivores including only PB (likelihood-ratio test, $\chi^2 = 18.706$, $df = 1$, $P < 0.001$, Fig. 5-6).

DISCUSSION

This study clearly showed that, due to functional dissimilarity, stoichiometric differences among detritivores played an important role in the effects of diversity on ecosystem function. Species of RB tended to consume litter with low C: P ratios or a high P concentration, and species of PB tended to consume litters uniformly, supporting prediction 1. Furthermore, higher detritivore richness that included both RB and PB increased the litter decomposition rate, supporting prediction 2.

Effects of diversity on decomposition by detritivores

Our results showed that litter decomposition was affected by not only species richness, variation of body size and number of feeding type, but also stoichiometric combination (Table 5-4, Fig. 5-4, 5-5). Therefore, our data admitted the importance of factors that focused on previous studies (i.e. body size and feeding type), and supplied new perspective (i.e. stoichiometric diversity) in the study of relationships between detritivores diversity and litter decomposition. Our results showed feeding preferences depending on the stoichiometry of detritivores (Fig. 5-3). In particular, the consumption of litter by species belonging to RB was significantly affected by litter nutritional properties, especially P concentration, while species classified as PB were not affected

(Table 5-3). As detritivores in freshwater ecosystems maintain low N: P ratios in their bodies relative to detritus (Evans-white et al. 2005; Small and Pringle 2010), they might respond to P availability rather than N availability. These differences in feeding preference might produce complementarity effects on litter decomposition rates. In fact, the decomposition rates in microcosms containing two species of detritivores differed significantly between microcosms containing one RB and one PB and microcosms containing two RB or two PB (Fig. 5-6). Many studies have tested whether litter decomposition rates were affected when species were lost from systems (Gessner et al. 2010). However, the effects of species loss differ among studies (Jonsson and Malmqvist 2000; McKie et al. 2008, 2009). The differences in these results may have been caused by differences in the stoichiometry of the detritivores used. It seems likely that the C : N : P body ratios of the detritivores used were similar in studies in which diversity effects were not detected. When a consumer eats a food, consumers with nutrient-rich bodies might increase the C : P ratio of their body. However, because their C : nutrient ratio increases only moderately (DeMott et al. 1998), it can be beneficial to search for high-quality resources rather than to remake the body.

When we examine the effects of diversity on decomposition, we must consider the diversities of both leaf litter and detritivores, as these will interact in the decomposition process. The quality of leaf litter varies widely among species (e.g., C : N: P ratios, lignin content), and may affect the degradation ability of detritivores (Gulis et al. 2006; Hladyz et al. 2009). Additionally, differences in litter quality affect microbial colonization, and change the palatability to detritivores (Kominoski et al.

2007; Jabiou and Chauvet 2012). We manipulated both leaf litter and detritivore diversity, and found that the feeding preferences of some detritivores among litter species were caused by their stoichiometric differences, affecting decomposition rate. Thus, the diversities of both detritivores and litter are important when considering the effects of diversity on decomposition rate.

Effects of litter diversity on microbial decomposition

Our results also showed that fungal biomass on leaf litters and the microbial decomposition rates of litters were affected by litter diversity (Fig. 5-2). There were no significant differences in fungal biomass or decomposition rates among litter species in SL; however, the biomass and decomposition rates of *A. japonica* and *S. obassia* in ML were significantly higher than the others (Fig. 5-2). Therefore, the mixing of leaf litter might affect the decomposition rate by altering the microbial biomass on leaf litters. The N concentration of *A. japonica* and P concentration of *S. obassia* were higher than those of other species (Table 5-1). Nutrient compounds of litter species rich in nutrients may be translocated to other types of litters (Hättenschwiler and Gasser 2005, Schimel and Hättenschwiler 2007). Therefore, the balances of utilizable C, N and P might be changed by litter mixing, thus affecting the fungal biomass and microbial decomposition rates of *A. japonica* and *S. obassia* in ML. This implied litter-mixing effect on fungal biomass and microbial decomposition rate would be influenced by the types of nutrients found at high levels in each litter species. Previous studies have reported positive, negative or no effects of litter diversity on decomposition in streams

and forest floors (Gessner et al. 2010). The differences in these results might be explained in part by the stoichiometric diversity of the litter assemblages.

Some studies have shown that litter mixing had non-additive effects on macroinvertebrate community structure in streams (Gulis et al., 2006; Kominoski and Pringle 2009). In the future, we must determine whether these effects arise from stoichiometric differences among detritivores. Litter decomposition rate is a key process not only in stream ecosystems but also in terrestrial ecosystems. Many studies have verified the effects of diversity on decomposition (Gessner et al. 2010). Some have shown that body size is an important functional trait that facilitates differential modes of resource use (Bardgett and Wardle 2010). However, no previous studies have focused on the stoichiometric diversity of detritivores. The species richness of detritivores is considerably higher on forest floors than in streams, and thus, the potential effects of detritivore stoichiometric diversity on decomposition might be greater and more complex in terrestrial systems. In fact, Gonzalez et al. (2011) showed that the P contents of terrestrial arthropods vary widely among species. Therefore, our findings might also be applicable to terrestrial communities, highlighting the role of the stoichiometric diversity of detritivores as a driver of ecosystem functioning.

Table 5-1 C, N and P concentration, C: N and C: P ratio and body mass (mean $\pm$ 1 SE)

of the body tissues of each species of detritivore.

	Functional feeding group	C concentration (mg/g)	N concentration (mg/g)	P concentration (mg/g)	C:N	C:P	Bodymass (mg)
<i>Jesogammarus jesoensis</i>	Shredder	495.92 (11.18)	128.09 (10.77)	10.40 (1.90)	5.47 (0.77)	45.17 (5.15)	1.89 (0.19)
<i>Sternomoera yezoensis</i>	Shredder	598.13 (18.92)	124.63 (12.18)	11.34 (1.78)	4.85 (0.52)	53.17 (9.50)	0.79 (0.09)
<i>Goerodes satoi</i>	Shredder	531.18 (8.74)	114.19 (7.05)	11.12 (2.01)	5.00 (0.29)	46.19 (6.21)	1.07 (0.11)
<i>Nemoura</i> sp.	Shredder	561.11 (23.20)	69.56 (8.90)	5.01 (0.81)	7.92 (0.60)	108.04 (19.66)	0.68 (0.05)
<i>Amphinemura</i> sp.	Shredder	574.80 (18.45)	70.17 (9.91)	4.81 (1.01)	8.24 (0.35)	123.90 (6.89)	0.71 (0.01)
<i>Cincticostella nigra</i>	Collector-gatherer	517.97 (34.81)	91.37 (10.46)	4.33 (0.51)	5.60 (0.34)	121.85 (7.82)	0.63 (0.20)

Table 5-2 Chemical properties (mean $\pm$ 1 SE) of leaf litter before and after the experiment. MSL, leaf litter placed in microcosms comprised a single species of litter; MML, leaf litter placed in microcosms comprised a mixture of four litter species.

	C concentration (mg/g)	N concentration (mg/g)	P concentration (mg/g)	C:N ratio	C:P ratio	Lignin (mg/g)
Before the experiment						
<i>Quercus crispula</i>	541.92 (11.87)	9.45 (0.36)	0.34 (0.02)	57.57 (1.53)	1622.98 (128.76)	185.27 (5.23)
<i>Carpinus cordata</i>	554.92 (17.49)	17.55 (0.80)	0.55 (0.02)	27.80 (0.65)	1164.27 (91.51)	230.48 (3.82)
<i>Alnus japonica</i>	474.64 (5.76)	21.19 (0.92)	0.78 (0.09)	22.80 (0.70)	644.71 (68.49)	224.20 (2.95)
<i>Styrax obassia</i>	430.78 (16.00)	16.74 (2.95)	1.90 (0.26)	32.37 (6.11)	265.93 (30.39)	206.50 (6.97)
After the experiment						
(MSL)						
<i>Quercus crispula</i>	446.08 (4.60)	8.23 (0.34)	0.31 (0.01)	54.63 (2.65)	1446.68 (57.86)	197.75 (13.89)
<i>Carpinus cordata</i>	449.05 (4.97)	15.14 (0.63)	0.50 (0.03)	29.83 (1.09)	903.75 (49.71)	251.77 (4.78)
<i>Alnus japonica</i>	501.68 (19.46)	26.94 (1.51)	0.91 (0.02)	18.70 (0.36)	550.18 (10.05)	185.65 (13.70)
<i>Styrax obassia</i>	444.40 (10.44)	10.47 (0.66)	1.68 (0.06)	42.91 (1.88)	722.96 (20.91)	245.72 (4.25)
(MML)						
<i>Quercus crispula</i>	476.79 (6.00)	8.46 (0.56)	0.29 (0.02)	57.38 (4.10)	1692.60 (138.30)	222.82 (3.85)
<i>Carpinus cordata</i>	519.90 (9.42)	21.07 (0.51)	0.72 (0.06)	24.73 (0.80)	738.58 (63.98)	260.06 (9.67)
<i>Alnus japonica</i>	553.42 (12.07)	30.23 (0.80)	1.06 (0.09)	18.34 (0.51)	542.81 (53.73)	295.93 (19.79)
<i>Styrax obassia</i>	479.62 (15.03)	14.03 (0.20)	1.24 (0.03)	34.20 (1.11)	386.49 (6.27)	291.05 (9.93)

Table 5-3 The most parsimonious models for explaining the variance in decomposition rates among litter species in MML into which was placed a species of detritivores. The modeling was conducted using a generalized linear model (GLM) with stepwise selection based on AIC. I estimated factors that affect the feeding preference of each detritivore species.

Species name	Experimental variable	Coefficient (Estimate $\pm$ SE)	P-value	AIC
<i>Jesogammarus yesoensis</i>	Intercept	0.0251 $\pm$ 0.001	< 0.001	-565.88
	P concentration (mg/g)	0.0051 $\pm$ 0.009		
<i>Sternomoera yezoensis</i>	Intercept	-0.0218 $\pm$ 0.0182	< 0.001	-607.40
	Fungal biomass (mg/g)	0.0012 $\pm$ 0.0003		
	C:P ratio	-0.000016 $\pm$ 0.0000084		
	P concentration	0.0106 $\pm$ 0.0043		
<i>Goerodes satoi</i>	Intercept	0.0190 $\pm$ 0.0027	< 0.001	-544.74
	Fungal biomass (mg/g)	0.00025 $\pm$ 0.00013		
	P concentration (mg/g)	0.0040 $\pm$ 0.0019		
<i>Nemoura</i> sp.	Intercept	0.022 $\pm$ 0.0025	0.148	-557.55
	Fungal biomass (mg/g)	0.00014 $\pm$ 0.000097		
<i>Amphinemura</i> sp.	Intercept	0.0271 $\pm$ 0.00235	0.679	-565.61
	Fungal biomass (mg/g)	0.000038 $\pm$ 0.000009		
<i>Cincticostella nigra</i>	Intercept	0.0257 $\pm$ 0.0012	0.297	-610.17
	C:P ratio	-0.0000012 $\pm$ 0.000001		

Table 5-4 Relationships between decomposition rate per detritivore metabolic capacity in microcosms and each explanatory variable. Relative importance of each of the explaining variable were also showed.

Explanatory variable	Relative importance of variable	Estimate	z-value	P value
Stoichiometric combination	0.999	0.104 ± 0.003	34.375	$P < 0.001$
Variation of body size	0.998	0.130 ± 0.002	68.700	$P < 0.001$
Number of detritivore species	0.500	0.101 ± 0.002	44.25	$P < 0.001$
Number of feeding type	0.269	0.098 ± 0.007	14.47	$P < 0.001$
Species combination	0.0005	0.112 ± 0.005	21.707	$P = 0.06$

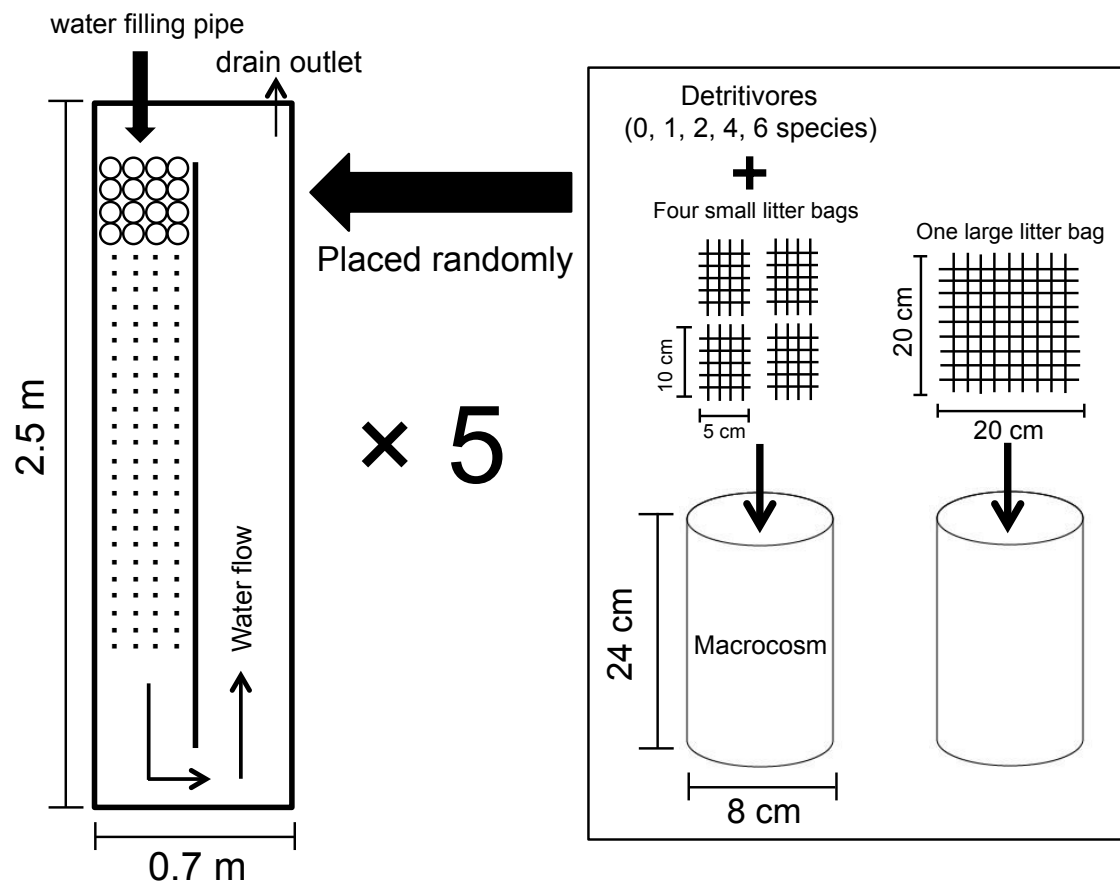


Fig. 5-1 Schematic diagram of our experimental system.

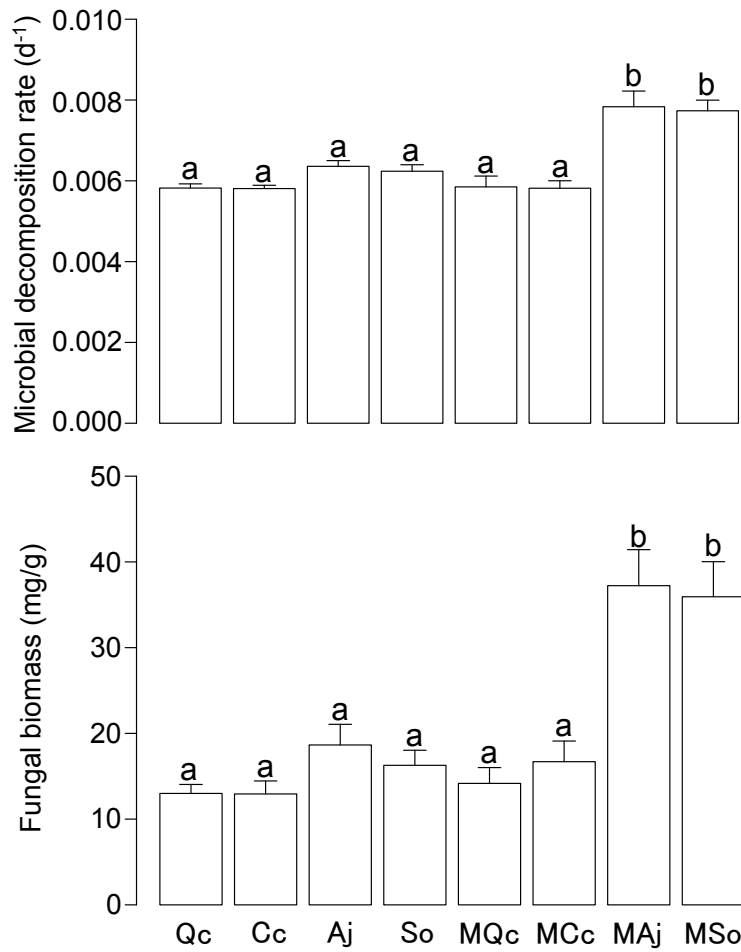


Fig. 5-2 Microbial decomposition rate of leaf litters per day and fungal biomass on leaf litters (mean $\pm$ 1 SE). Qc: *Quercus crispula* in microcosms have only single species litter (MSL), Cc: *Carpinus cordata* in MSL, Aj: *Alnus japonica* in MSL, So: *Styrax obassia* in MSL, MQc: *Quercus crispula* in microcosms have mixed all four litter species (MML), MCc: *Carpinus cordata* in MML, MAj: *Alnus japonica* in MML, MSo: *Styrax obassia*. Significant differences between vegetation types are denoted by different letters ($P < 0.05$).

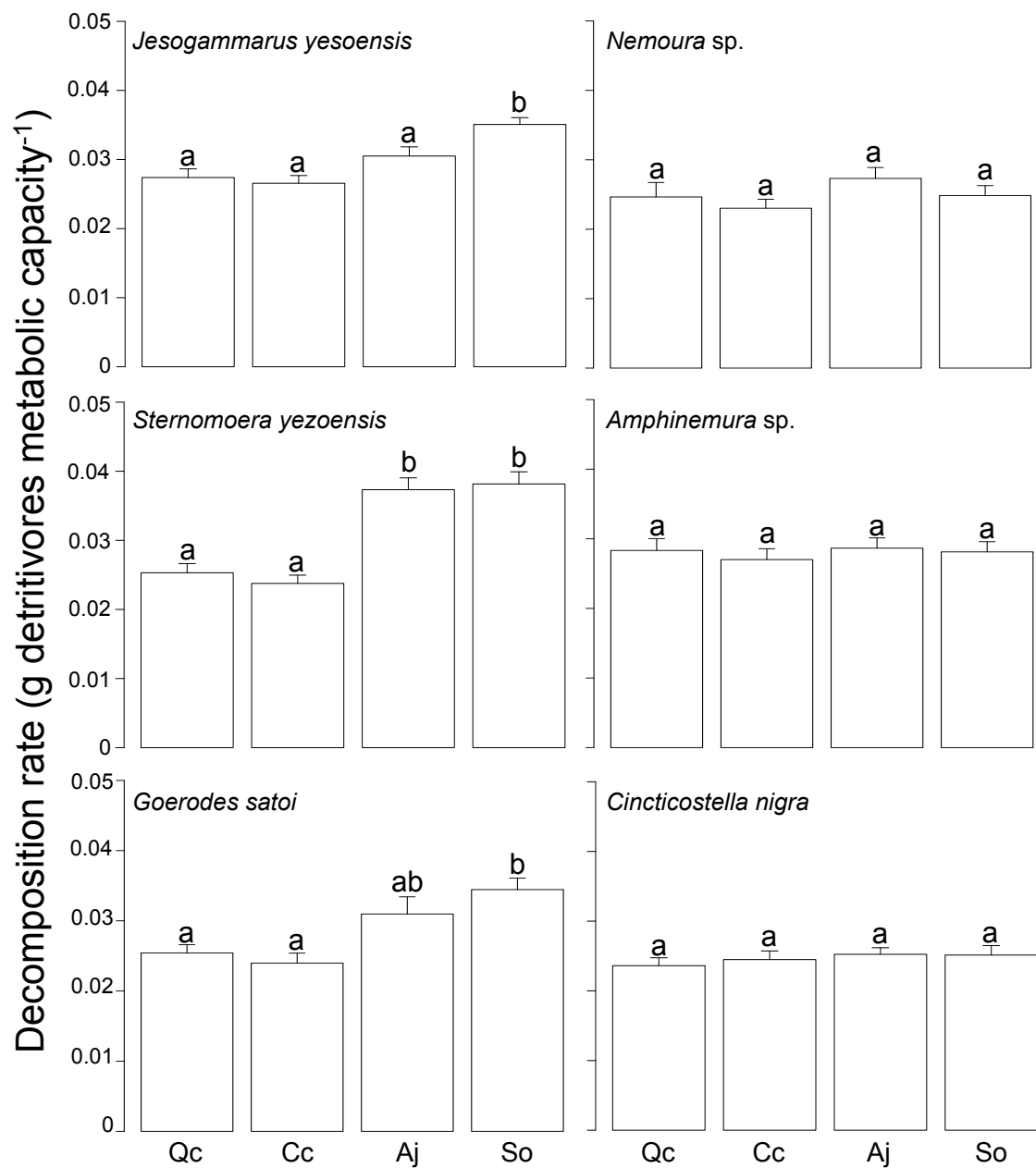


Fig. 5-3 Decomposition rate per detritivore metabolic capacity in microcosms placed a species detritivores among litter species in MML. Mean and standard errors (+1SE) are shown. Significant differences between species of litters are denoted by different letters ($P < 0.05$). Mean and standard errors (+1SE) are shown.

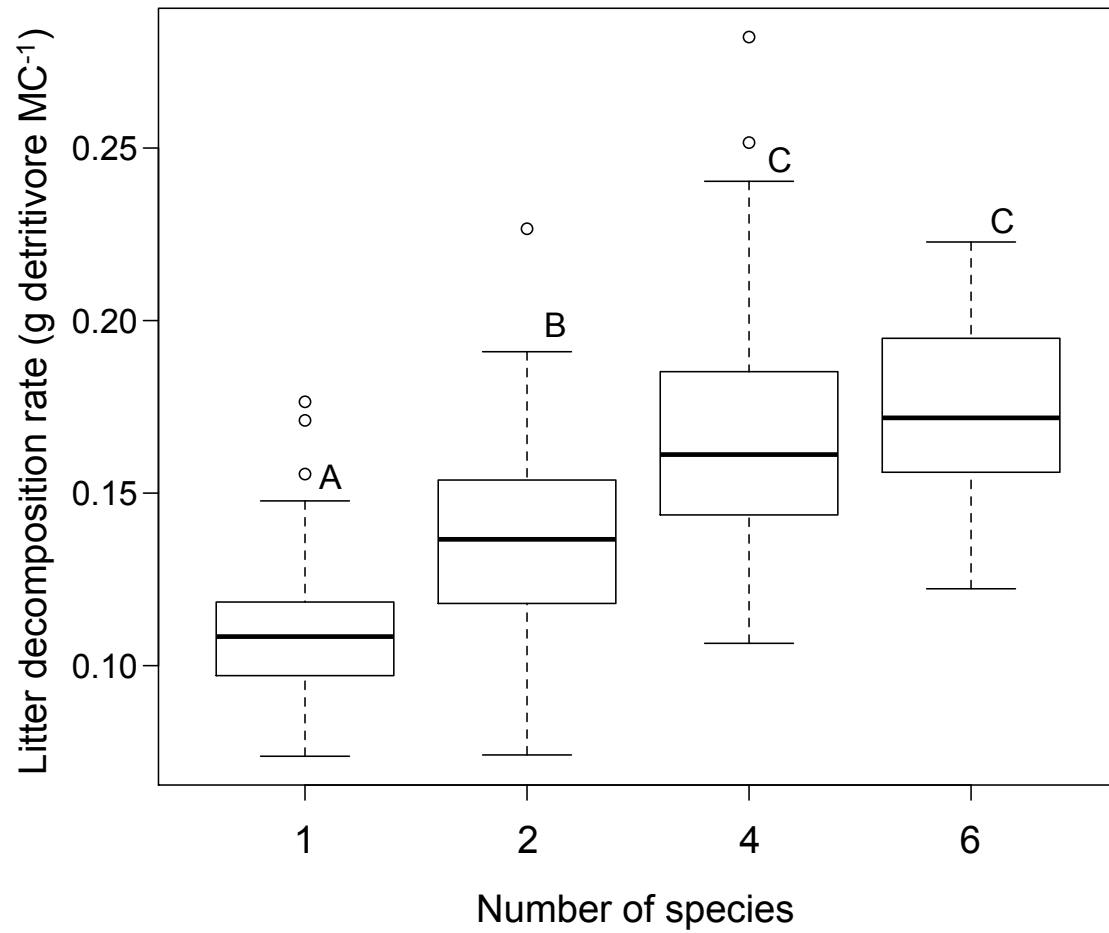


Fig. 5-4 Litter decomposition rates per detritivores metabolic capacity (MC) among number of detritivores species. Significant differences between vegetation types are denoted by different letters (post-hoc pairwise likelihood ratio tests, $P < 0.05/4$).

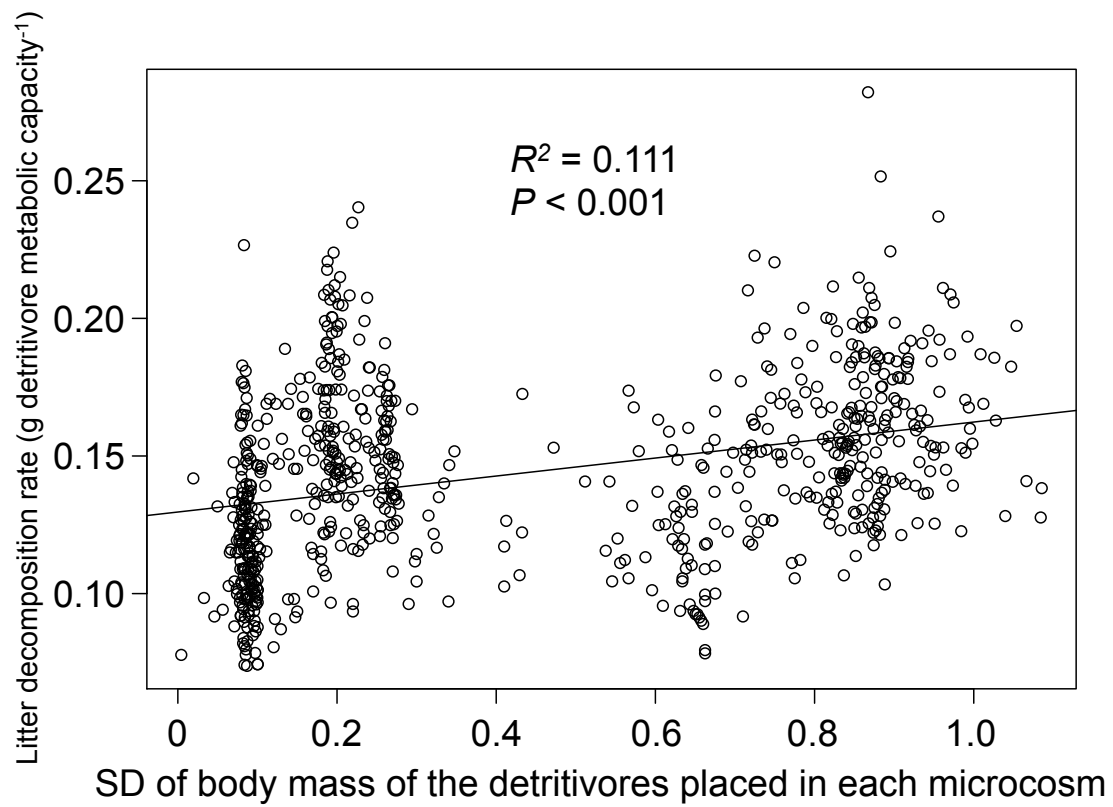


Fig. 5-5 Relationship between standard deviation (SD) of the detritivores placed in each microcosms and litter decomposition rate per detritivore metabolic capacity.

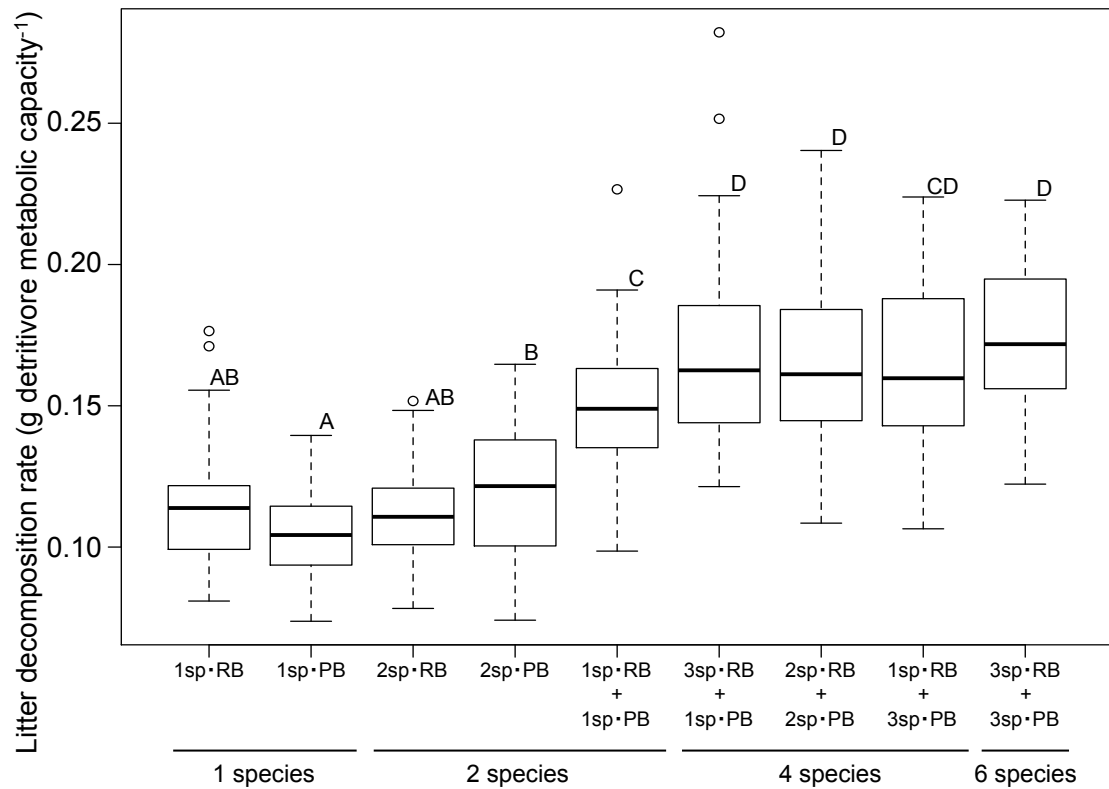


Fig. 5-6 Total litter decomposition rates (i.e. sum of the decomposition rate each species of litter) per detritivore metabolic capacity among stoichiometric combination in each microcosm. Significant differences between stoichiometric combinations are denoted by different letters (post-hoc pairwise likelihood ratio tests, $P < 0.05/9$). NRB means detritivores included a group have nutrients rich body, NPB means detritivores included a group have nutrients poor body. -sp means the number of NRB or NPB placed in the microcosms.

Chapter 6

General discussion

In this thesis, I considered relationships between resources and consumers in streams from stoichiometric theory. Ecological stoichiometry is defined by Sterner & Elser (2002) as the balance of multiple chemical substances in interactions and processes. Then the studies using stoichiometric theory have become widespread in terrestrial, marine and freshwater ecosystems (Makino et al. 2003; Hessen et al. 2004; Moe et al. 2005).

Effects of the resources stoichiometry on its consumers

To consider the relationships between consumers and basal food resources such as producers and litters, we have frequently focused on significant imbalances between the two (Cross et al. 2003; Hessen et al. 2013). Several studies have linked elemental stoichiometry in resources to growth, reproduction, biomass and community structure of consumers (Sterner & Elser 2002; Sardans et al. 2012; Hessen et al. 2013). These studies were frequently conducted in lake ecosystem using zooplanktons and snails (e.g. Elser et al. 2000a; Frost et al. 2010). For example, some studies defined that C : P and N : P stoichiometry in resource are important viewpoints, because elevated growth and reproduction rates are linked to elevated demands for P for the synthesis of P-rich ribosomal RNA (Elser et al. 2000a; 2000b; 2000c; Sterner & Elser 2002; Liess & Hillebrand 2005; Fink & Von Elert 2006). And Hessen et al. (2007) showed N : P ratio in the resources and growth rate are linked via the intimate connections between P allocation to ribosomes and N allocation to protein synthesis. In stream ecosystem, there are studies that demonstrated the stoichiometry of resources affected growth,

reproduction and feeding mode and community composition of the invertebrates and consequently the ecosystem functions (Sardans et al. 2012). Some studies showed nutrient availability in the resources altered the growth rate of the consumers (Rosemond et al. 1993; 2000; Stelzer & Lamberti 2001; Bowman et al. 2005; Christian et al. 2008; Kendrick & Benstead 2013), and consequently affect organic matter flows between trophic levels and decomposition rate (Cross et al. 2006; 2007; Davis et al. 2010b).

Many studies have focused environmental factors those alter C : N : P stoichiometry of resources, and demonstrated the effects on consumers (e.g. Urabe et al. 2002; Cross et al. 2006; Yu et al. 2010) (Allow B in the Fig. 1-1). However, generalizations of the observations in another ecosystems were only half done. For example, in lake ecosystem, several studies demonstrated that light intensity affected growth rate of the primary consumers through alterations of the producers' stoichiometry (Urabe et al. 1996; 2002; Hillebrand 2005). Although C : N : P stoichiometry in producers are also affected by light intensity in stream ecosystems (Fanta et al. 2010), the indirect effects of light intensity on consumers had not been demonstrated (but see Hill et al. 2010). In Chapter 2, I demonstrated light intensity was a factor that alter C : N : P stoichiometry of periphyton and affect growth and reproduction of a grazer using artificial channels (Ohta et al. 2010). Because light intensity in stream is easily changed due to riparian forest structure and dynamics, light intensity actually affect growth and reproduction of grazers in natural ecosystem. However in my experiment, I ignored many factors that present in natural ecosystem,

such as behavior of grazer and presence of predators. In the future, these studies have to be examined in natural stream ecosystem.

Furthermore, changes in subsidiary resources should be important factors those alter stoichiometry of resources in stream ecosystem. Previous studies showed the changes in subsidiary nutrient are produced by changes in terrestrial vegetation and human activities (e.g. Smith et al. 1999; Fukuzawa et al. 2006; Tokuchi & Fukushima 2009). Especially, the effects of forest management and atmospheric depositions on the water chemistry of streams have been studied throughout the world, and many studies reported the nutrient release from terrestrial to stream ecosystem can be changed by the clear-cut logging and planting in the catchments (Palviainen et al. 2004; Löfgren et al. 2009; Tokuchi & Fukushima 2009), the agricultural fertilization (Turner et al. 1998; Goolsby et al. 1999) and the increase in atmospherically-derived nutrients (Kroeze & Seitzinger 1998; Chadwick et al. 1999). Many studies have estimated effects of nutrient release from terrestrial to stream on relationships between resources and stream invertebrates using stoichiometric theory (Allan & Castillo 2007; Cross et al. 2007). In consequences, experimental nutrient additions in stream ecosystems have been conducted throughout the world (e.g. Cross et al. 2005; 2006; 2007; Davis et al. 2010a; 2010b; Rosemond et al. 2010; Connolly & Pearson 2013). These studies showed nutrient enrichment decreased C : N and/or C : P ratios in food resources, and increased the litter decomposition rates (Robinson & Gessner 2000; Rosemond et al. 2010; Connolly & Pearson 2013), the growth rates of grazer (Rosemond et al. 1993; 2000; Christian et al. 2008), the material flows among trophic levels (Cross et al. 2007) and

the average size structures of invertebrates (Davis et al. 2010a; 2010b).

Additionally, although various minerals (e.g. calcium, magnesium and potassium) are also essential elements for many organisms, previous studies mainly focused on changes in C : N : P stoichiometry. Despite these minerals in stream are supplied from terrestrial ecosystem, effects of subsidiary minerals were rarely estimated in aquatic ecosystem (but see Hessen et al. 2000). In Chapter 3, I focused the importance of the vegetation in catchment on stream crustaceans through calcium concentration in the litters (Ohta et al. in press). Because there is no study that demonstrated subsidiary calcium from litter of the catchment vegetation, this is an important finding to discuss the linkage between terrestrial and aquatic ecosystem. Additionally, because organisms tissues contain various elements, calcium : another nutrient stoichiometry should be considered. In fact, since body tissues of stream crustaceans contain not only calcium but also P in high concentration (Vrede et al. 1999a; 1999b), and calcium and P stoichiometry in the environmental materials become important (He & Wang 2009). P in stream ecosystem is also supplied from terrestrial ecosystem, and the supply depends on terrestrial condition such as differences of base rock and vegetation. Therefore, when we consider the effects of subsidiary resources on the recipient ecosystem, their stoichiometry of numerous elements should be focused on. Recently, some researchers are proposing that inputs of subsidiary resources are temporally variable and the seasonal timing of the supplied subsidy is important to the recipient ecosystem (Anderson et al. 2008; Marczak & Richardson 2008; Sato et al. unpublished data). The amount of subsidiary calcium might be changed by the supply of

fresh litter, and stoichiometry in resources might be temporally changed, although I ignored the importance in this study.

There may be many factors those affect ecosystem function and community in stream other than change in stoichiometric imbalances of resources, such as physical disturbance and presence of predators (Allan & Castillo 2007; Dodds 2010). Few studies showed the effects of factors those alter stoichiometry of resources might be modified some factors such as water temperature and physical disturbance (Gafner & Robinson 2009; Kendrick & Benstead 2013). In Chapter 4, I demonstrated whether the effects of nitrogen enrichment on litter decomposition and invertebrate colonization were altered by light intensity. This is a new perspective to estimate the effects of nutrient enrichment on the litter decomposition and the invertebrate communities in a stream. In the future, it is necessarily to discuss with these factors, and comprehensive considerations.

Ecological implication of stoichiometric differences among consumers

Previous studies detected the stoichiometry of consumers vary among species and with the surrounding environment (Sturner and Hessen 1994; Elser et al. 1996, 2000a; Sturner and Elser 2002; Raubenheimer and Simpson 2004; Evans-White et al. 2005; Frost et al. 2010; Persson et al. 2010; Small et al. 2010). Some studies detected links between lower C : P and N : P stoichiometry and higher growth and/or reproduction rates in terrestrial plants (Niinemets & Kull 2005; Elser et al. 2003; Cernusak et al. 2010; Zhang & Han 2010) and animals (Kay et al. 2006; Apple et al. 2009;

Visanuvimol & Bertram 2010) and also in aquatic animals (Darchambeau et al. 2003; Anderson et al. 2005). Difference in C : N : P body composition ratios among organisms also alter the exclusion rate in stream and lake ecosystems (Elser & Urabe 1999; Vanni et al. 2002; Boersma & Elser 2006; Jensen et al. 2006; Torres & Vanni 2007; Pilati & Vanni 2007; Shimizu & Urabe 2008; Christian et al. 2008). Although several studies have found no clear relationships between body size and N : P ratio (Dantas & Attayde 2007; Bertram et al. 2007; Martinson et al. 2008), a study has reported a connection between large body size and low N : P body ratio (Méndez & Karlsson 2005). Therefore, difference in stoichiometry among consumers would have important consequence for ecosystem functions linking the relationships between resources and consumers. In addition, the stoichiometric differences among consumer species also have some relationships with feeding behavior of the consumers such as feeding activity (Plath & Boersma 2001; Schatz & McCauley 2007) and resource choice (Sterner & Elser 2002; Schmitz 2010). The effects of stoichiometric differences among consumers on ecosystem function have never been verified yet, although the difference in feeding behavior might affect ecosystem function such as decomposition rate (Belovsky 1997; Bernays 1998). In Chapter 5, I demonstrated the importance of detritivores' stoichiometric diversity on litter decomposition rate. This finding means stoichiometric theory can become an angle to examine the relationship between biodiversity and ecosystem function. Furthermore, my finding has to be applied to another ecosystems, because the positive relationship between biodiversity and ecosystem function was reported in grassland (Schmitz 2010), soil (Gessner et al. 2010)

and marine (Emmerson et al. 2001; Solan et al. 2012) ecosystems. For example, biodiversity of herbivores have a positive effect on herbivory rate in terrestrial and marine ecosystem (Srivastava & Vellend 2005; Brandt et al. 2012). In addition, some studies reported terrestrial herbivores have nutrient rich body feed selectively high-quality plant tissue to maintain body stoichiometry (Belovsky 1997; Bernays 1998). To consider biodiversity and herbivory rate, it may be possible to combine with stoichiometric diversity among herbivores. Therefore, stoichiometric approaches may be useful tools to solve these problems which were propounded in ecology (Woodward 2009; Schmitz 2010).

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