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Phosphorus Uptake in *Rhodomonas salina* (Wislouch) and Its Effect on Allocation and Elimination in *Acartia tonsa* (Dana)

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by

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ABSTRACT

PHOSPHORUS UPTAKE IN *RHODOMONAS SALINA* (WISLOUCH) AND ITS EFFECT ON ALLOCATION AND ELIMINATION IN *ACARTIA TONSA* (DANA)

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Old Dominion University, 2010
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Phosphorus is a key element in important biochemical compounds, such as RNA and phospholipids, and can become limiting in a variety of marine systems. The uptake of phosphorus into biochemical fractions (protein, low molecular weight (LMW) compounds, lipid, polysaccharide and nucleic acid) in *Acartia tonsa* fed ^{33}P –labeled *Rhodomonas salina* was examined. *R. salina* was cultured on two variations of one media that in one case contained phosphorus in balance and the other out of balance with relation to other standard f/2 components. The P-balanced (PB) media had a N:P ratio of 24.5, which is higher than that found in the Redfield N:P ratio of 16. The P-imbalanced (PI) media, on the other hand, had an N:P of 245.3. PB *R. salina* incorporated more ^{33}P into their cells than did the PI *R. salina* in all but the LMW compounds fraction. ^{33}P was incorporated faster into all biochemical fractions in the tissue of *A. tonsa* when fed PB *R. salina*. The ^{33}P uptake kinetics of *A. tonsa* were rapid in both treatments, but always higher when the copepods were fed PB *R. salina* during the standard uptake experiment and variable among biochemical fractions in the modified uptake experiment. The elimination of the ^{33}P signal from biochemical fractions was investigated, with pre-labeled *A. tonsa*, during exposure to different feeding environments (i.e., artificial seawater without phosphorus, *R. salina*, *Thalassiosira weissflogii*). ^{33}P was released more rapidly in the fed rather than in the unfed treatments, indicating that phosphorus turnover

in copepods is directly dependent on the quantity of assimilated phosphorus. However, the feeding history of *A. tonsa*, initially fed on food of different elemental composition, did not affect elimination rates in any food environment. This study provides insights into how the physiology and homeostatic control of algae and copepods dampen fluctuating concentrations of inorganic nutrients for higher trophic levels.

This thesis is dedicated to my family and friends who have been there every step of the way and never let me falter.

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TABLE OF CONTENTS

Page

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

INTRODUCTION

Nutrient limitation in phytoplankton negatively affects zooplankton by leading to decreased growth (Müller-Navarra, 1995; Rothhaupt, 1995), decreased egg production (Weers and Gulati, 1997; van Donk et al., 1997) and difficulties molting (Sterner et al., 1993). Copepods can have their growth restrained either by an insufficient amount of food or by feeding on a nutrient-deficient/imbalanced diet. Nutrients in various biochemical components can affect food webs either as a result of the elemental ratio (i.e., among C, N, P, S, etc.) at which they are supplied or in terms of their biogeochemical characteristics. Nutrients can further be separated into those that are related to specific compounds (i.e., specific amino acids or fatty acids) and those that are related to bulk properties such as proteins, lipids and carbohydrates (Anderson et al., 2004). Food quality is generally described by its effect on the animals consuming it and can be determined by studying the difference in growth of predators on prey items with differing elemental composition, independent of quantity (Sterner and Schulz, 1998; Mayzaud et al., 1998), as quality can remain an issue at low (Boersma and Kreutzer, 2002) and high prey abundance (Urabe and Sterner, 1996). When presented with prey of diminished nutrient content, it is unclear how the animal will allocate the incoming nutrients. However, the growth rate hypothesis states that differences in an organism's elemental ratios of carbon, nitrogen and phosphorus are due to variable allocation of phosphorus to RNA, which is necessary for biomass growth through protein synthesis (Sterner and Elser, 2002). For example, if an organism was supplied with a lesser amount of phosphorus than what was required for growth and reproduction, it would have to choose between allocating the phosphorus to growth or to maintenance, a strategy

proposed by a model by Frost et al. (2005). Protein synthesis is a complicated process that involves many compounds and molecules containing both nitrogen and phosphorus. While phosphorus limitation thus provides a direct effect of reduced growth (Müller-Navarra, 1995; Rothhaupt, 1995), Hessen et al. (2007) demonstrated in their work with a planktonic rotifer, *Brachionus* that nitrogen limitation can also affect the phosphorus content.

While the biochemical make-up of organisms consists of various elements, phosphorus is of particular interest. In addition to nucleic acids, phosphorus is a key element in many other important cellular biochemical compounds, such as lipids and proteins in the forms of phospholipids and phosphoproteins, respectively, and as the universally important cell fuel adenosine triphosphate (ATP). Thus, when an animal is presented with limited quantities of a nutrient, the decision to allocate to different biochemical compounds reflects a prioritization of certain biochemical compounds. Determination of the hierarchy of allocation among biochemical pools will lead us to a better understanding of the underlying biochemical constraints of the metabolic machinery during times of limitation.

Extensive work, with regards to the effects of phosphorus limited prey on zooplankton, has already been done with the freshwater cladoceran, *Daphnia*. Adult *D. magna* fed high phosphorus cultured *Scenedesmus obliquus* (1.4 mg P/L) were denser and had higher weight-specific phosphorus content than those fed phosphorus-limited cultured *S. obliquus* (83.7 μg P/L) (Boersma and Kreutzer, 2002). Furthermore, Sterner and Schulz (1998) showed that *D. magna* and *D. galeata* grew substantially better on algae with higher phosphorus contents than on algae with lower phosphorus contents.

This result could be directly due to reduced phosphorus supply in the media or indirectly due to the algae's biochemical (i.e., polyunsaturated fatty acids - PUFAs) changes in response to lower phosphorus, like that shown by Gervais and Riebesell (2001). Their work with the diatom *Skeletonema costatum* demonstrated that initially the growth rate was not affected by the reduced storage of phosphorus, but when phosphorylated intermediates and nucleotides became reduced, growth rates were affected. Eventually, growth was halted entirely and phosphorus was directed to structural components (i.e., DNA, membrane lipids). This concept was further substantiated by studies in which zooplankton offered phosphorus-deficient algae showed reduced growth (Müller-Navarra, 1995) leading to smaller organisms (Weers and Gulati, 1997; Sterner et al., 1993). The aforementioned studies thus demonstrate a switch from growth to basic maintenance, as suggested by Gervais and Riebesell (2001).

Daphnia has been shown to survive on a diet of phosphorus-deficient algae, but it ultimately resulted in a reduction in growth rate (Sterner and Schulz, 1998; Gervais and Riebesell, 2001) and fecundity (van Donk et al., 1997) due presumably to difficulties assimilating nutrients from prey with increased cell wall thickness, since numerous undigested cells were found in the feces (van Donk et al., 1997). Thickening of the cell wall is a response to unfavorable ambient nutrient conditions in some algae, possibly leading to encystment (van Donk et al., 1997). In experiments with *D. galeata*, *Rhodomonas lacustris* and *Scenedesmus quadricauda*, Vrede et al. (2002) illustrated that differences in the RNA: DNA ratio can arise quickly, and that the RNA: DNA ratios were higher in treatments with higher P: C, which was to be expected as RNA is thought to be a significant pool of phosphorus in *Daphnia* (Sterner and Hessen, 1994). Similarly,

DeMott et al. (1998) demonstrated that juvenile *Daphnia* fed phosphorus-deficient (culture media: 6 mM P) algae had lower phosphorus content than those fed phosphorus-replete (culture media: 100 mM P) algae. The lower phosphorus content, in effect, should also lead to a reduced growth rate, which can be seen in results of Klein-Breteler et al. (2005), as well as those of Rothhaupt (1995), whose experiments were performed with marine copepods and freshwater rotifers feeding on nutrient-limited algae (Klein-Breteler et al., 2005: <0.01 μM P and <2.6 μM N; Rothhaupt (1995): 2 μM P and 40 μM N), respectively.

If two similar organisms are compared and one has a reduced requirement for a particular nutrient, the results may differ, as seen in Sterner and Schulz (1998). They found that *Diaphanosoma* and *Sinobosmina*, cladocerans with lower phosphorus contents than that of *Daphnia*, did not display reduced growth on algae with lower phosphorus content to the same extent as the *Daphnia* did; perhaps the phosphorus to carbon ratio required to satisfy their elemental balance was not as great. Keeping this result in mind, marine zooplankton has been found to have lower P: N ratios than freshwater zooplankton (Elser and Hassett, 1994). This could mean that marine zooplankton may be less susceptible to phosphorus limitation than their freshwater counterparts.

Some authors suggested that marine zooplankton assimilate elements based on the proportions supplied in their diet (Urabe, 1993; Reinfelder and Fisher, 1991), leading to the generalized "you are what you eat" argument (Sterner and Elser 2002). Additionally, studies such as Elser and Hassett (1994), found that marine seston was enriched in phosphorus compared to freshwater seston, which might have led to the perception that phosphorus is generally not limiting in marine systems (Sterner and Hessen, 1994).

However, there is some evidence that marine copepods might in fact be phosphorus limited as seen in work by Petrola et al. (2002) in the Baltic Sea (0.00-0.05 μM PO_4). In their study, several marine zooplankton species, including the calanoid copepod *Acartia* spp., were found to be phosphorus limited (Petrola et al., 2002).

Average Redfield ratios for dissolved inorganic carbon, nitrogen and phosphorus (C:N:P of 106:16:1) in seawater implies no nitrogen (as nitrate) or phosphorus (as phosphate) limitation of algal growth and thus "perfect" proportions for algal growth. This emergent property of seawater is considered the very result of homeostatic regulation of elements due to phytoplankton growth (Falkowski, 2004). Marine phytoplankton are expected to perform nutrient uptake in these same proportions during growth, which should be near their maximum rate in nutrient replete environments and release them back into the column during decomposition in the same proportions. While the concentrations of dissolved carbon, nitrogen and phosphorus in the world's oceans can vary considerably, the proportions between elements remain on average, and over large spatial and temporal scales, very similar. Some zooplankton can maintain their chemical composition, or be homeostatic, only within a narrow range of values when consuming foods with imbalanced elemental composition relative to their natural food (Elser and Urabe, 1999). Further support of this phenomenon is seen in the work of Tang and Dam (1999), who showed that *Acartia tonsa*, a fairly cosmopolitan species of zooplankton in coastal marine ecosystems, changes its elemental composition in response to differential nutrient input provided by food items, but also only within a limited range.

The intent of this study was to investigate the effect of nutrient imbalances of food algae, using the cryptophyte *Rhodomonas salina*, on the incorporation of radioactive

phosphorus into different biochemical fractions of the copepod *A. tonsa*. The rate of phosphorus incorporation and the shape of its uptake, as well as elimination kinetic curves for each fraction were studied in detail to determine the varying phosphorus pathways or pools for deposition (RNA, phospholipids, etc.). *R. salina* was selected for copepod rearing and incorporation because it is known to be a high quality food source (Veloza et al., 2006; Støttrup and Jensen, 1999; Støttrup et al., 1999). Both *R. salina* and *Thalassiosira weissflogii* were offered in the phosphorus elimination experiment as they both have been shown to be of high quality for copepods (Tang, 2005). The study aimed at determining any differential elimination rates produced by the use of the two algae. All facets of the following experiments were designed to determine biochemical shifts, if any, in *A. tonsa* solely due to the elemental and biochemical composition of *R. salina*.

Rhodomonas salina and *Acartia tonsa* were expected to take up ^{33}P according to Michaelis-Menten kinetics. Michaelis-Menten kinetics are characterized by a saturation curve demonstrating a rapid initial uptake, which is followed by a stabilization of values, indicating saturation (Fig. 1a). Kinetic parameters include maximum theoretical rate of ^{33}P incorporation (dpm_{max}) and the half saturation constant (K_m), which is the time required to incorporate half of the dpm_{max}. Additional parameters include dpm_t as the amount of radioactivity at time t and r as the instantaneous rate of ^{33}P elimination. The elimination of ^{33}P from the copepod tissue, once individuals were transferred into a non-radioactive environment, was expected to follow an exponential decay (Fig. 1b) as the copepods were being presented with unlabeled food.

Several hypotheses were investigated in a series of experiments using the

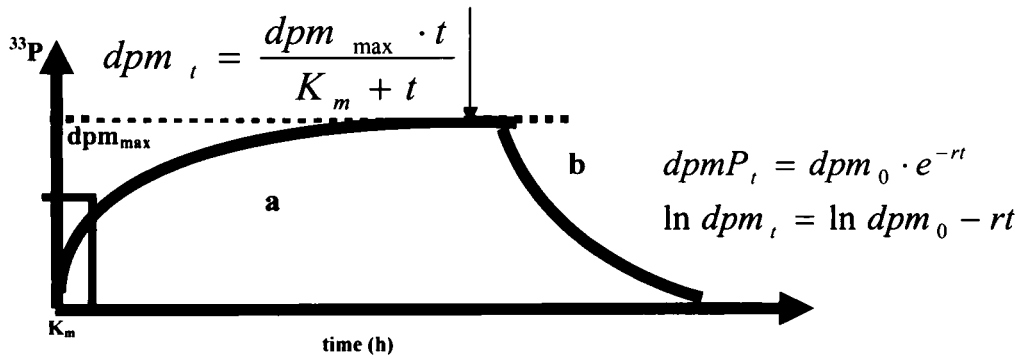

Fig.1. The theoretical time course of ^{33}P incorporation into the algae and copepods (Michaelis-Menten) (a), and the elimination of ^{33}P from copepods after transfer into unlabeled artificial seawater (exponential decrease) (b). Variables are dpm_{max}: maximum theoretical rate of ^{33}P incorporation; K_m: half saturation constant; dpm_t: amount of radioactivity at time t and r: instantaneous rate of ^{33}P elimination.

radioactive isotope ^{33}P as a tracer. The first several sets of hypotheses addressed the incorporation and allocation of radioactivity into the algae in a chemostat system. Culturing of the algae was performed with two different types of media, phosphorus-balanced (PB) and phosphorus-imbalanced (PI). The PB media was standard f/20-Si media with a N:P ratio of 24.5, which was near Redfield (N:P of 16.0). PI media was based on f/2-Si media with the exception of phosphorus at f/20 concentrations, which produced higher compound (i.e., trace elements, vitamins, etc.)/element:P ratios with a N:P ratio of 245.3. Keeping phosphorus constant and varying the other nutrients, in order to create nutrient imbalance in the prey, had two distinct advantages: first, phosphorus was available in exactly the same quantities in the two treatments. Therefore any changes in phosphorus allocation were due to nutrient ratios and not due to absolute availability of phosphorus. Second, since ^{33}P was used as the tracer, differential isotope dilution due to ^{31}P did not need to be accounted for when following the time kinetics of phosphorus uptake because the ratios between ^{31}P and ^{33}P remained the same. Radioactive phosphorus was added to all media in equal volumes and concentrations. Care was taken

to provide food at saturated concentrations in all experiments so that there was no food limitation effect (Møller and Nielsen, 2001).

Two groups of hypotheses regarding ^{33}P incorporation were tested in this study, one for the algae and one for the copepods:

a) *R. salina*:

1) ^{33}P is incorporated faster into the algal tissue in the P-balanced (PB) treatment than in the P-imbalanced (PI) treatment.

2) Incorporation rates of ^{33}P are faster into the low molecular weight (LMW) compounds, due to labile molecules (i.e., ATP), than into the protein, lipid and polysaccharide-nucleic acid fractions.

3) The P-balanced (PB) treatment algae incorporate more ^{33}P into all fractions than the P-imbalanced (PI) treatment algae.

b) *A. tonsa*:

1) The ^{33}P incorporation rates are faster into labile molecules (i.e., ATP, RNA, etc.) than into the protein, lipid or polysaccharide fractions.

2) ^{33}P incorporation rates are faster into all fractions in body tissue when fed on the P-balanced (PB) treatment algae than when fed on the P-imbalanced (PI) treatment algae.

3) More ^{33}P is incorporated into all fractions of the body tissue when fed on the P-balanced (PB) treatment algae than when fed on the P-imbalanced (PI) treatment algae.

Based on phosphorus's varying importance or content between different cellular compounds and thus biochemical fractions, it was also critical to examine the effect that

feeding history had on allocation to those compounds. Zooplankton often experience patchiness of both food quantity and quality with some patches of prey providing just enough nutrients to survive, while others offer nutrients above and beyond what they require. The question then becomes how zooplankton will respond when greater amounts of nutrients become available in a new patch. It may be, initially, whatever nutrient was limiting is retained and then eventually released at a reduced rate. Or perhaps, once realizing the nutrient levels are high, the zooplankton initiate rapid uptake. This study attempted to determine the effects of pre-feeding regimes (P-imbalanced or P-balanced algae) on elimination rates of radioactive phosphorus from copepods suddenly presented with prey of standard nutrient content. In this experiment two different prey items, a cryptophyte and a diatom, were used to determine their effects on elimination rates compared to starving zooplankton. Both *Rhodomonas salina* and *Thalassiosira weissflogii* are thought to be high quality food prey for *Acartia tonsa*. Similarly, differences in prey types offered may also lead to a differential elimination rate of radioactive phosphorus based on the prey's nutritional status. Elimination rates in various fractions reflect the turnover rates of specific nutrients within the organism. Thus, the final set of hypotheses, regarding the elimination of radioactive phosphorus from the copepod tissue, was:

1) Copepods pre-fed phosphorus-imbalanced (PI) treatment algae are more conservative with their body phosphorus and eliminate it at a lower rate than those pre-fed phosphorus-balanced (PB) treatment algae.

2) Fed copepods eliminate ^{33}P faster than starving ones, as new incoming phosphorus replaces the resident one.

3) The type of diet (*R. salina* and *T. weissflogii*) influences the elimination rates of phosphorus.

4) Regardless of feeding history, the low molecular weight (LMW) compound fraction has the fastest elimination rate of ^{33}P in comparison to the more structural protein, lipid or polysaccharide-nucleic acid fractions.

METHODS

Adult female *Acartia tonsa* from the culture collection of the University of Copenhagen were reared for these experiments. The copepods were grown on the cryptomonad *Rhodomonas salina*, obtained from the Bigelow Laboratory culture collection (CCMP 1319), at saturation levels, based on fluorometric readings greater than 2000. In order to precisely control the elemental nutrient composition of the experimental water, both the food algae and copepods were cultured, or reared, in artificial seawater as per Kusk and Wollenberger (1999) (Appendices I-III) with salinity of 30.0 (Sullivan and McManus 1986, Stearns et al. 1989, Medina and Barata 2004). Both cultures were maintained in an environmental chamber kept at a constant temperature of exactly 20°C, which has been shown by various authors to result in rapid development (Miller et al., 1977; Gillooly, 2000), fast hatching (Landry, 1975), good hatching success (Sullivan and McManus, 1986) and was suitable for general culturing of copepods (Stearns et al., 1989; Medina and Barata, 2004; Besiktepe and Dam, 2002; Colin and Dam, 2002). The experimental food source for the copepods during the ^{33}P uptake experiments was *R. salina*. Prior to the start of the experiments, *R. salina* was grown on f/2 without silica (f/2-Si) medium, while the diatom *Thalassiosira weissflogii* (CCMP1049 from Bigelow Laboratory), used only in the elimination experiments, was grown on f/2 media (Appendix IV, Guillard and Ryther, 1962; Guillard, 1975).

In preparation for the experiment, artificial seawater was prepared as previously mentioned, but without the addition of phosphorus. It was imperative to prepare the seawater without phosphorus, so that all phosphorus inputs were experimental. This P-deplete medium was distributed among the various bottles in the experimental system,

autoclaved and amended with nutrients based on their volume and treatment (see Phase I and Appendix IV).

Experimental layout

As the present study contained multiple experiments, a generalized overview is presented in Fig. 2. There was one preparatory phase (I), which established the experimental media, and three experimental phases (II-IV) that consisted of two phases incorporating ^{33}P and the final phase eliminating ^{33}P. The subsequent sections reveal the explicit details within each phase.

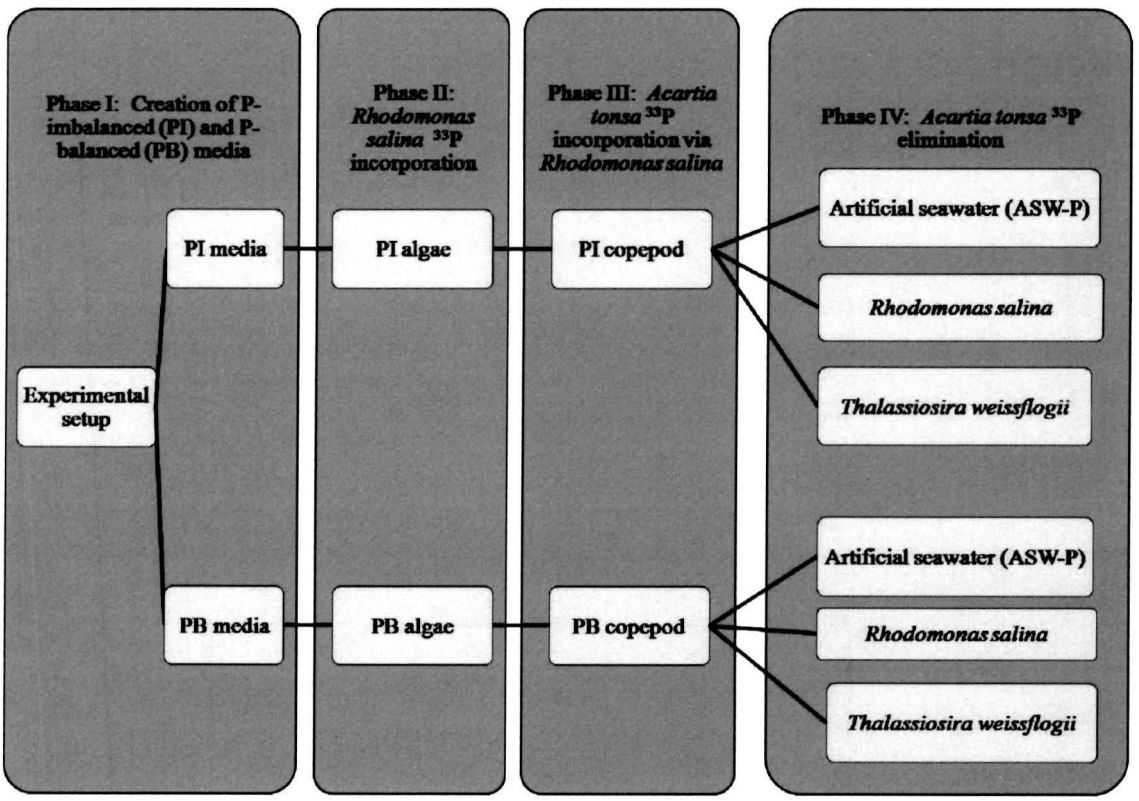

Fig. 2. Simplified layout of the complete four-phased experiment. See phase headings for specific details.

Phase I: Experimental media setup for ^{33}P uptake kinetics of Rhodomonas salina

The experimental media for algae culturing was based on f/2 media by Guillard and Ryther (1962) and Guillard (1975). The P-imbalanced (PI) media contained nutrients at standard f/2-Si concentrations, except phosphorus, which was at f/20-Si concentrations (Appendix IV). P-balanced (PB) media, on the other hand, contained all nutrients at f/20-Si concentrations (Appendix IV). Thus, in both treatments, the same concentration of phosphorus was added. All nutrients were delivered sterilely using a syringe equipped with a 0.2-μm filter.

For the experimental setup, a series of autoclaved flasks, containers and tubing were used, as well as a peristaltic pump to provide a chemostat system (Fig. 3). The 4-l nutrient reservoir flasks (Fig. 3a) were connected to the smaller 1 l culture flasks, or aspirator bottles, (Fig. 3b) with PharMed® BPT tubing at the top via a silicon stopper. A Gilson Miniplus® 3 peristaltic pump (Fig. 3d) delivered algae via the PharMed® BPT tubing and VWR International® polypropylene and polyethylene straight connectors from the culture flasks to the daily collection flasks or to the copepod feeding chambers (Fig. 3c). The tubing was routed through the peristaltic pump (Fig. 3d), which resulted in delivery of 250 ml of nutrients or algae to the respective containers per day. The algae were kept in suspension by gentle stirring using a Fisher Scientific lab disc (Fig. 3e) and magnetic stirrer bars. The chambers were set up in an environmental chamber at 20°C and received constant light for the entire length of the 240-hour experiment at 225 $\mu E\ m^{-2}\ s^{-1}$.

Once the chemostat system was established (Fig. 3), radioactive phosphate, as orthophosphate $H_3{}^{33}PO_4$, was added to each of the four bottles (2 nutrient reservoir

flasks, Fig. 3a and 2 culture flasks, Fig. 3b), resulting in ^{31}P and ^{33}P concentrations of 3.6 μM and 2.8 X 10^{-5} μM, respectively. Final total concentration of phosphorus was 3.6 μM for both treatments and 883.0 μM N in the P-imbalanced (PI) and 88.3 μM N for the P-balanced (PB). These concentrations resulted in N:P ratios of 24.5 and 245.3 for PB and PI, respectively. Therefore, isotope dilution in the algae and the copepod tissues occurred to the same extent in each treatment, and did not require a dilution correction factor. The bottles were quickly swirled after each addition to immediately disperse the radioisotope.

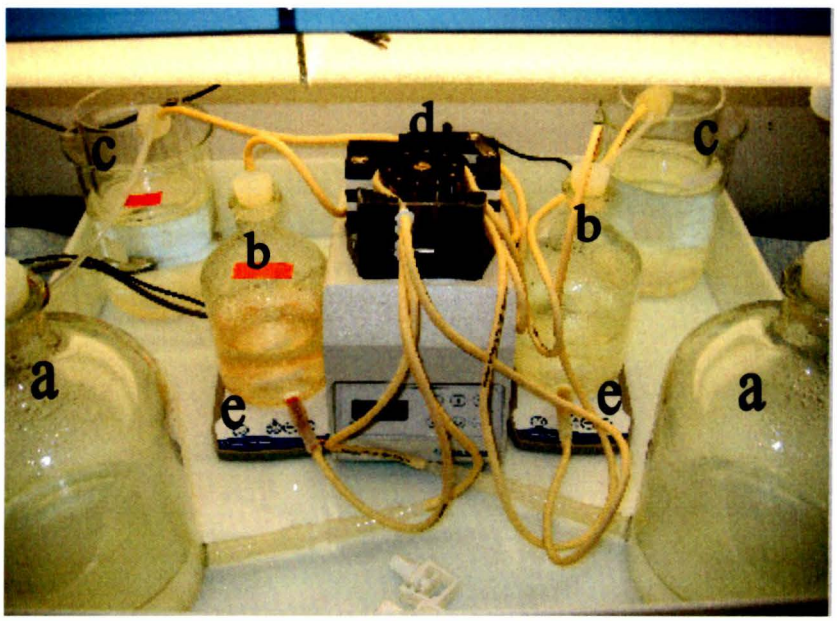

Fig. 3. Experimental setup showing positions of equipment. The experimental setup consisted of nutrient reservoir flasks (a), culture flasks (b), feeding chambers containing the copepods (c), peristaltic pump (d), and magnetic stirrer bars (e).

Phase II: Experimental setup for ^{33}P uptake kinetics of Rhodomonas salina

Forty milliliters (ml) of a dense *Rhodomonas salina* culture was added to each of the culture bottles and mixed again by swirling. The peristaltic pump was then activated to a flow rate of 250 ml/day, which was half of the 500 ml volume contained in the 1 l

flask (Fig. 3b) and forced a 0.5 per day growth rate upon the algae after some initial fluctuations. The 0.5 per day growth rate was selected to allow for maximum algae availability for the copepods, without flushing the entire system every 24 hours. The cultures were then left undisturbed for five days, or 10 divisions (Olesen et al., 2005), and were considered to be uniformly labeled at that time (Wang et al., 1996). A 170 ml of outflow sample was collected daily for analyses of the algae. The rest of the algae were drained into the copepod feeding chambers (Fig. 3c).

To investigate the ^{33}P uptake and labeling pattern for the *R. salina*, and to verify steady state conditions at the time of copepod addition daily samples of *R. salina* for total radioactivity (^{33}P dpm), particulate phosphorus, particulate carbon, Chl *a* and cell abundance (Fig. 4) were taken from the collection flask with volumes listed in Table 1. The collection flask was swirled before each sample was taken to ensure homogeneity of suspension. Samples from the collection flask were filtered onto pre-combusted 25 mm Whatman© glass fiber filters using a hand pump with filter tower for the analysis of particulate phosphorus, particulate carbon, Chl *a* and ^{33}P radioactivity. Liquid samples were transferred to scintillation vials and either preserved with acid-Lugol's for enumeration of cell abundance or received 4 ml of scintillation cocktail. The samples were removed with a 1, 10 or 25 ml Fisherbrand® polystyrene disposable pipette. Duplicate samples were taken from each culture on all samplings days.

Algae samples

The daily *Rhodomonas salina* samples were analyzed for particulate phosphorus, particulate carbon, Chl *a*, cell abundance, particulate radioactive phosphorus (non-fractionated), dissolved phosphorus, dissolved radioactive phosphorus and particulate

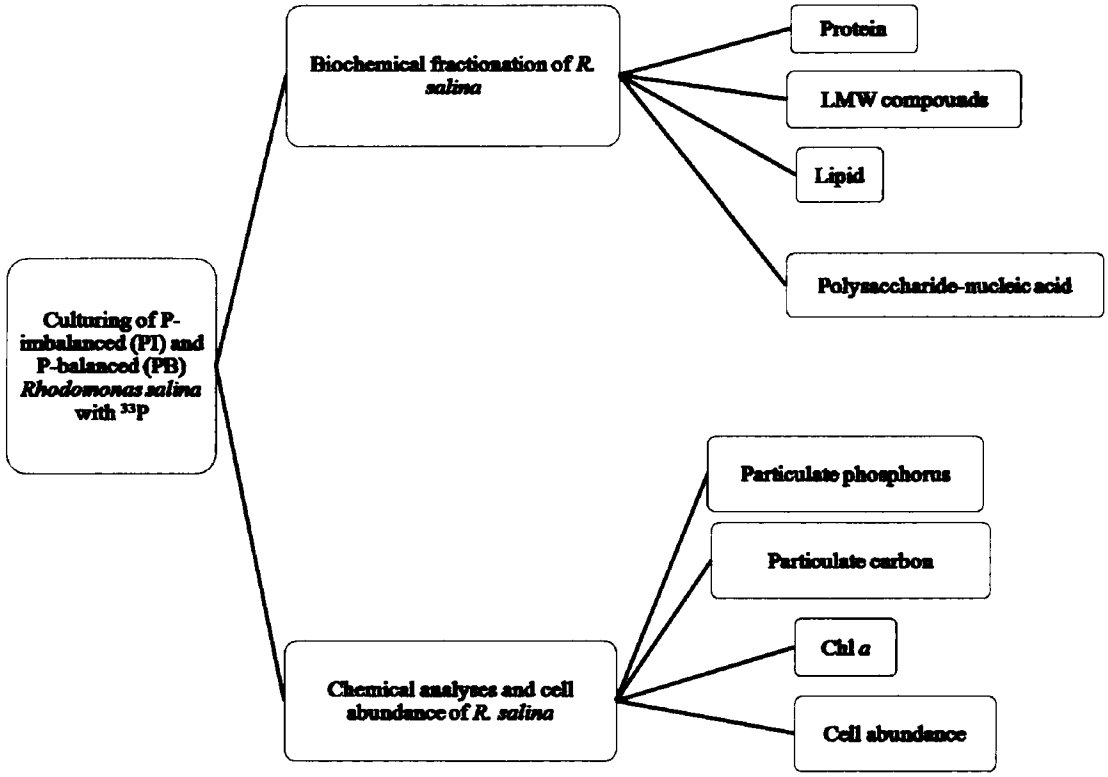

Fig. 4. Flowchart of related *Rhodomonas salina* samples and analyses during the ^{33}P incorporation experiment.

radioactive phosphorus (fractionated) (Table 1). The volumes, depending on the sample, were filtered in a 20 ml filter tower with a hand vacuum pump. Dissolved samples were taken from the filtrate before any rinsing of the tower occurred to prevent dilution of phosphorus. A 0.5 ml sample of filtrate, containing dissolved radioactive phosphorus, was deposited into a scintillation vial to which 4 ml of scintillation cocktail was added, well mixed and immediately placed in a Beckman™ LS-5000TD scintillation counter. Then, 20 ml of the filtrate, containing dissolved phosphorus, was added to a scintillation vial and was immediately frozen (-20°C) until analysis. During filtering of algae samples, the tower was rinsed twice with 2 ml of 0.17 M sodium sulfate ($NaSO_4$) (Appendix VII) for particulate phosphorus, particulate radioactive phosphorus (non-

Table 1
Summary of samples taken during algae/copepod ^{33}P incorporation experiments and copepod ^{33}P elimination experiment with their respective volumes (ml) or number of individuals per sample. N/A = not applicable.

Sample	*Rhodomonas salina* ^{33}P incorporation experiment	*Acartia tonsa* ^{33}P incorporation experiment	*Acartia tonsa* ^{33}P elimination experiment
Particulate phosphorus (ml)	25	25	25
Dissolved phosphorus (ml)	20	20	20
Particulate ^{33}P (ml)	1	1	N/A
Dissolved ^{33}P (ml)	0.5	0.5	N/A
Standard biochemical fractionation algae (ml)	25	25	N/A
Chl *a* (ml)	5	5	5
Cell enumeration – Lugol's (ml)	1	1	1
Standard biochemical fractionation – copepods (individuals per filter)	N/A	4-9	4-5
RNA biochemical fractionation – copepods (individuals)	N/A	7-10	N/A

fractionated) and particulate radioactive phosphorus (fractionated), and phosphorus free artificial seawater was used for Chl *a* samples and particulate carbon. All filter samples were stored in the freezer (-20°C), except particulate radioactive phosphorus (non-fractionated), to which 4 ml of scintillation cocktail was added and the vials were placed in a Beckman™ LS-5000TD scintillation counter with the dissolved samples and counted using ^{33}P protocols with custom quench curves. The samples were read for 20 minutes each or to the precision of 2-sigma =0.5, whichever occurred first. Samples for cell abundance were placed into scintillation vials and preserved with acid-Lugol's.

Phase III: Experimental setup for ^{33}P uptake kinetics of Acartia tonsa

After five days of ^{33}P uptake and incorporation into *Rhodomonas salina*, the cells were considered to be fully labeled (i.e., the isotopic equilibrium among biochemical fractions was achieved; Wang et al., 1996) and the copepod experiment commenced. The analytical scheme of the copepod ^{33}P incorporation experiments is shown in Fig. 5. Approximately 200 adult, lab-reared female *Acartia tonsa* were randomly assigned into two beakers containing artificial seawater without phosphorus (ASW-P). These two populations were then rinsed several times with ASW-P in an attempt to remove any residual phosphorus (^{31}P) from the original copepod culture water. Finally, in a 2000 ml beaker (Fig. 6a), with 600 ml of ASW-P, the copepods were transferred into a 10 cm diameter and 20.25 cm length Plexiglas® tube with a 150-μm mesh bottom (Fig. 6b). The stands on the bottom of the Plexiglass® tubes (Fig. 6c) created a space below the mesh screen in which old algae, fecal pellets and eggs could settle out. This physical separation prevented coprophagy (Lampitt et al., 1990), egg cannibalism (Dagg, 1977) and the recycling of nutrients associated with these processes (i.e., sloppy feeding). The

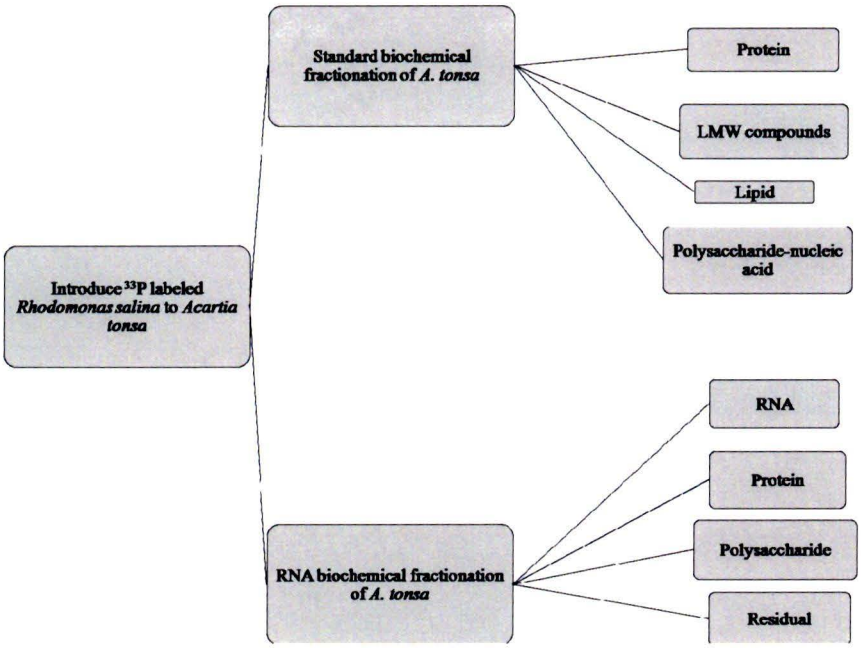

Fig. 5. Flowchart of *Acartia tonsa* experimental procedure and sample analysis during the ^{33}P incorporation experiments.

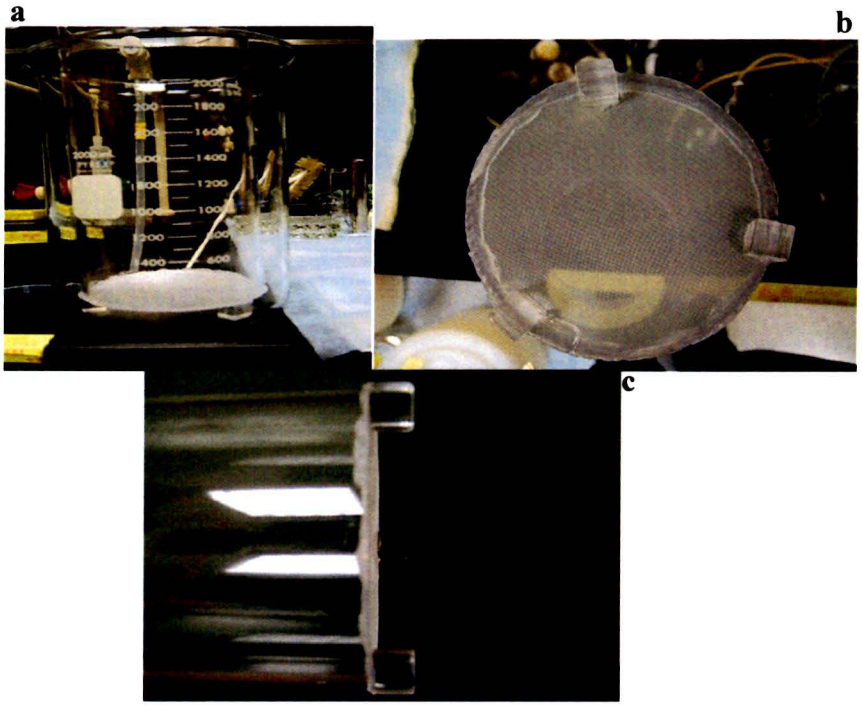

Fig. 6. Photos of experimental feeding setup and equipment. Experimental beaker with feeding chamber inside (a); close-up of the bottom of the tube (i.e., feeding chamber) with a 150 µm mesh and stand attachment (b); side-view close-up of the bottom of the tube (i.e., feeding chamber) (c).

beakers were situated to allow the culture tubing to deliver *R. salina* into the Plexiglas® copepod feeding chambers (Fig. 3c). During sampling the Plexiglas® tube was lifted to concentrate the copepods in a small volume for pipetting. In addition to subsamples of copepods for the ^{33}P incorporations, the culture tubing was removed from the copepod feeding chambers and connected to Pyrex® Erlenmeyer flasks to obtain samples for daily algae radioactivity (^{33}P dpm), particulate phosphorus, particulate carbon, Chl *a* and cell abundance samples, which represented what the copepods had been feeding on. These samples gave insight into the nature of the ^{33}P incorporation pattern.

The experimental copepods were examined for physical condition using a Corning™ 9 well glass plate and an Olympus™ SZ30 dissecting microscope. Healthy copepods, from each treatment, were placed into a 600 ml rinsing beaker containing a 150 μm mesh cup and ASW-P, where they were rinsed three times with ASW-P and then placed into a final beaker also containing ASW-P. Here, the copepods were left for two hours to allow for gut evacuation (Kiørboe and Tiselius, 1987). The gut evacuation helped ensure that the radioactivity measured in the copepod was based on actual ^{33}P incorporated into *A. tonsa* tissue and not residual radioactive algal material in the gut.

After the two hour evacuation, copepod health was again assessed. A filter was placed on top of the filter stand, moistened with ASW-P and a seal was created with the hand pump. A 6" Pasteur pipette was used to remove nearly all the water in the well thereby transferring minimal amounts of water with the copepods. The copepods were then removed from the wells using a modified 6" Pasteur pipette. The pipette had most of its tip broken off to increase the diameter of the tip by ca 3-fold and was flamed to remove the rough edges. This modification created an opening large enough not to

damage the animal, but not too large to take up excess water. Once the desired number of copepods was on the filter, the filter was removed, folded, placed into aluminum foil and frozen at -20° C. This process was duplicated for both treatments at the end of each time point.

Phase IV: Experimental setup for Acartia tonsa ^{33}P elimination

An overview of the entire ^{33}P elimination experiment from labeling of copepods to analysis is illustrated in Fig. 7. For the ^{33}P elimination experiment, two populations of 190 adult, lab-reared female copepods were obtained from the lab culture and treated as stated above in the Phase III for ^{33}P incorporation into *Acartia tonsa* body tissue. However, daily samples of *Rhodomonas salina* and *A. tonsa* were not taken during this part of the experiment; instead the copepods were allowed to feed undisturbed for five days on radio-labeled algae.

Once labeled, the copepods were removed and rinsed three times with ASW-P. To determine a baseline for radioactivity, an initial sample of copepods was taken at time 0 from each pre-fed treatment. Both populations of copepods, i.e., those fed P-imbalanced (PI) and P-balanced (PB) *R. salina*, were divided into three subgroups of 50-53 copepods each. The subgroups consisted of copepods kept in artificial seawater without phosphorus and without food (ASW-P), and copepods fed either on unlabeled *R. salina* or unlabeled *Thalassiosira weissflogii*. *R. salina* and *T. weissflogii* were chosen as they are both considered good food sources for *A. tonsa* (see Introduction). The utilization of both algae was to determine if one elicited a faster elimination rate due to variable nutritional value. A system of 500 ml beakers with Plexiglas® tubes 7 cm in diameter and 30.5 cm in length with 150 μm bottom mesh, similar to that used in the

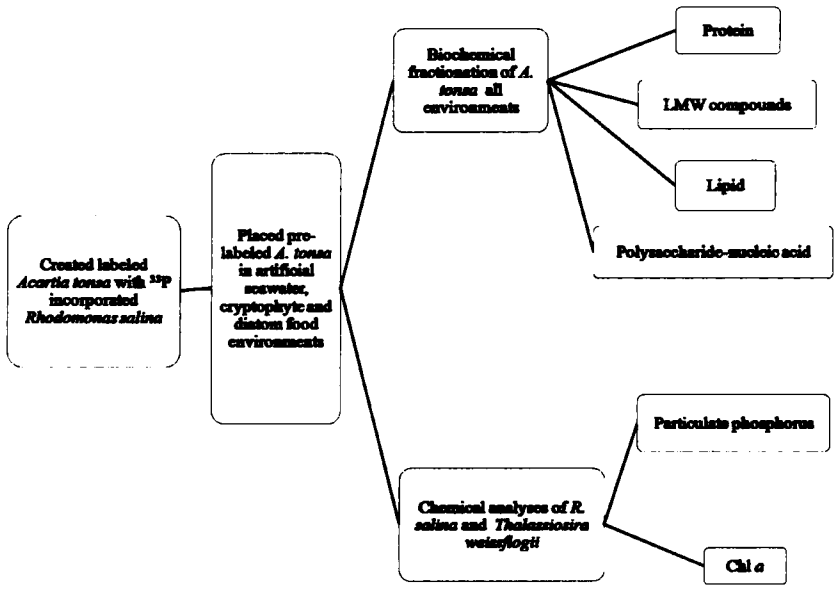

Fig. 7. Flowchart of *Acartia tonsa* experimental procedure and sample analyses during the ^{33}P elimination experiment.

uptake kinetics experiment (Figs. 6b-c), was used in this experiment (Fig. 8). At daily intervals, algae samples and subsamples of copepods (5-10 per filter) were taken, and the tubes were swirled to re-suspend any settling or settled algae. Copepods were rinsed three times with ASW-P and set aside for ~2 hours to allow for gut evacuation. Finally, copepod health was determined and either the copepods were placed on a filter or into the waste beaker.

Chemical analysis: Particulate phosphorus/dissolved orthophosphate

Particulate and dissolved phosphorus (as orthophosphate) were analyzed using the ammonium molybdenum method outlined by Solorzano and Sharp (1980) and Parsons et al. (1984). Prior to the analysis, scintillation vials and centrifuge tubes were acid washed and triple rinsed with Milli-Q water to eliminate outside sources of phosphorus. For particulate phosphorus, filters were removed from the freezer, placed into the acid

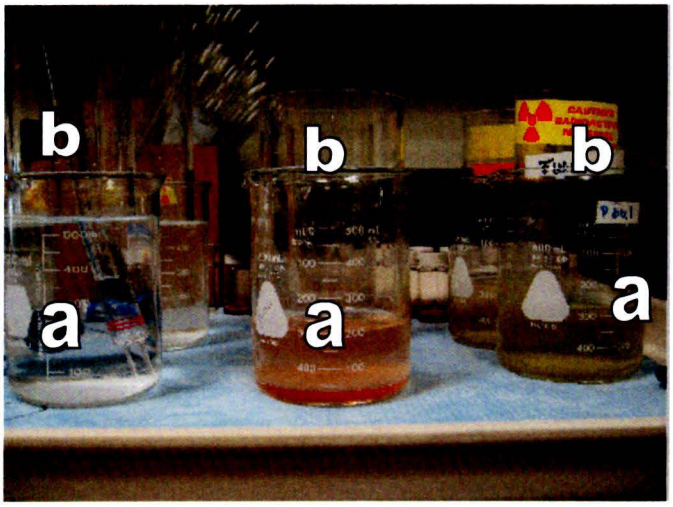

Fig. 8. Experimental setup for ^{33}P elimination experiments. Experimental beakers (a) and feeding chambers (b) are (left to right): artificial seawater (no food), *R. salina* and *T. weissflogii*.

washed vials and 2 ml of 0.017 M magnesium sulfate ($MgSO_4$) (Appendix VII) was immediately added. These vials, without caps, were placed into a drying oven at 60°C for 2.5-3.5 hours or until the filters were dry. Once cooled, the vials were covered with aluminum foil and placed in a muffle furnace at 450°C for 6 hours to burn off organic carbon. The vials were again cooled, 5 ml of 0.2 M hydrochloric acid (HCl) (Appendix VII) was added to each, then they were capped tightly and placed into the drying oven at 80-90°C for 30 minutes. As the vials cooled, a mixed reagent, blanks and phosphorus standards were prepared (Appendix VIII). Liquid and filters were removed from the vials and placed into acid washed 15 ml Fisherbrand® polypropylene centrifuge tubes. The vials were rinsed with 5 ml of Milli Q water to remove any residual liquid or particles and were also poured into the centrifuge tubes. One ml of mixed reagent was added to all centrifuge tubes and they were briefly vortexed. Then, the tubes were placed into a centrifuge and spun at 2,000 rpm for 5 minutes.

The supernatant was read in 1 cm polystyrene cuvettes in a Hitachi U-2001 spectrophotometer at 885 nm against reagent blanks using Milli Q water. Transferring of samples from the centrifuge tube to the cuvette consisted of removing approximately 1.5-2.0 ml of sample from the tube using a disposable polystyrene pipette and rinsing the cuvette with new sample. Then, approximately 3 ml of the same sample was placed in the cuvette with the transfer pipette and read by the machine. The dissolved phosphate samples (20 ml each) were thawed, processed by adding 2 ml of the mixed reagent (Parsons et al., 1984) and read. Absorbance was measured at 885 nm (see above).

Chemical analysis: Particulate carbon and nitrogen

In preparation for particulate carbon and nitrogen analysis, samples were removed from the freezer and placed in a drying oven at 40°C for 48 hours. The dried filters were folded and encased in thin aluminum circles, which were then deposited into a pelletizer to compress the aluminum cylinder. The samples were run in a Europa 20/20 mass spectrometer equipped with an automated nitrogen and carbon analyzer for gas, solids and liquids (ANCA-GSL) (Mulholland and Bernhardt, 2005) that had been calibrated with N_2 and CO_2 reference gasses and standard curves of known amounts of ammonium sulfate and sucrose (Bernhardt, 2009 personal communication).

Chemical analysis: Chlorophyll a

The Chl *a* samples were analyzed using direct fluorescence measurement (Welschmeyer 1994) against a purchased pure Chl *a* standard from Sigma ® Corporation. The Chl *a* samples on the GF/F filters were extracted in 10 ml of 90 % acetone at 4°C overnight in 15 ml Fisherbrand® polypropylene centrifuge tubes. The next day, the samples were vigorously vortexed and centrifuged at 1,380 g for 10 minutes. After

centrifuging, the tubes were removed, the samples were taken using a transfer pipette, deposited into clean test tubes and read in a Turner™ TD700 fluorometer, where 90% acetone served as the blank.

Chemical analysis: Standard four fraction biochemical fractionation

The standard biochemical fractionation protocol was based on Bligh and Dyer (1959), Li et al. (1980) with modifications provided by Rivkin (1985) and Bochdansky et al. (1999). The changes between protocols were with regards to volumes, times and temperatures. The standard biochemical fractionation of the algae and copepod samples resulted in four fractions: protein, low molecular weight (LMW) compounds, lipid and polysaccharide/nucleic acid (poly-na). The LMW compounds are usually defined as those compounds with molecular weights less than 1,000 daltons (Loh et al., 2004), however, they were defined operationally as the water-soluble fraction after Bligh and Dyer (1959) extraction. A simplified schematic of the standard biochemical fractionation protocol is presented in Fig. 9. Using a filtration manifold, filtration towers were loaded with new filters on top and 15 ml glass graduated centrifuge tubes were positioned below to catch the filtrate in the 250 ml vacuum flasks, which were attached to a vacuum pump modified with foot control. Sample filters were placed into eprouvets with the addition of 2.5 ml of a 1:2 chloroform/methanol mixture. However, the copepod samples were macerated using either a wide glass rod in an eprouvet (early samples) or a tissue grinder (later samples). The solution was then vortexed vigorously for a minute to ensure homogeneity of the sample. The eprouvets were placed into a styrofoam container with crushed ice and the solution extracted for 20 minutes. The solution, including filter, was decanted into the filter tower. An additional 2 ml of the 1:2 chloroform/methanol

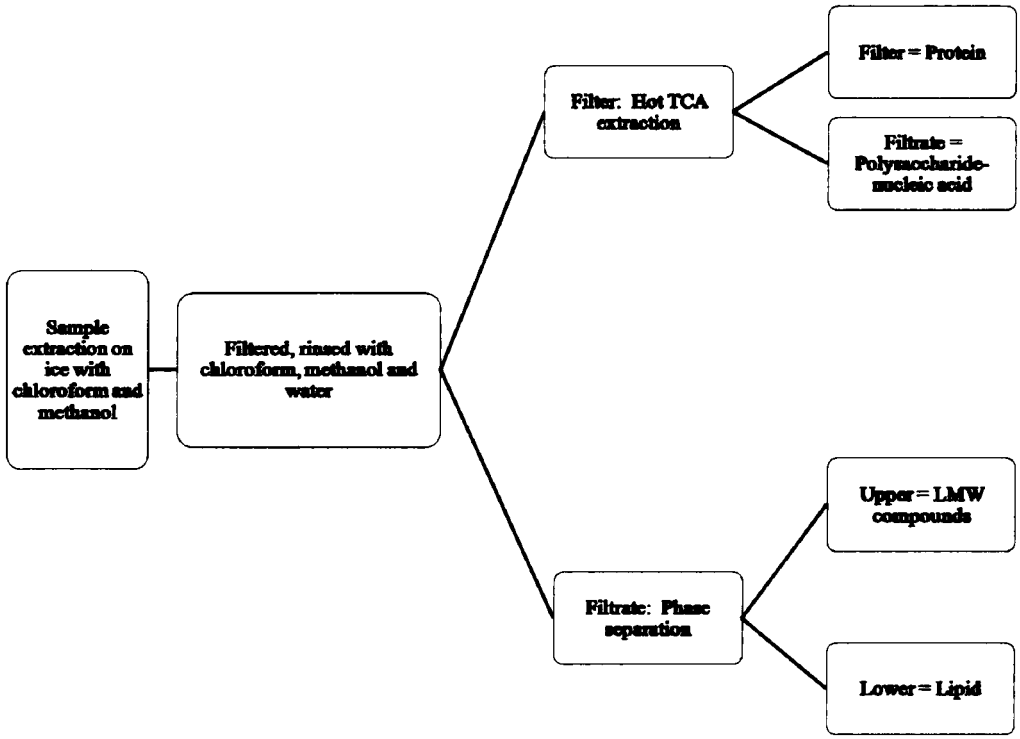

Fig. 9. Simplified overview of standard biochemical fractionation process. The process is identical for both algae and copepods with the exception that the copepods were macerated before extraction. The process was performed on several copepods captured on one filter.

mixture was dispensed into the empty eprouvet, to serve as a rinse, and was added to the tower. The vacuum pump was switched on, once the liquid disappeared, and a glass rod was gently pressed onto each filter to expel any remaining liquid. The glass rod was rinsed between treatment samples (PI or PB) to prevent contamination. The eprouvet was rinsed with 1.5 ml of pure chloroform and briefly vortexed before the solution was added to the tower. Then, the same eprouvet was rinsed twice with 1.5 ml of Milli Q water, which was then deposited into the tower. Again the liquid was removed via vacuum pump and the glass rod used to remove residual liquid from the filter. Finally, with the vacuum running, the filtration towers were removed and the filters were dislodged from the stand and placed into scintillation vials where 4 ml of 5% trichloroacetic acid (TCA) was added. These vials were tightly capped and transferred to 95°C pre-heated

aluminum-heating block with wells slightly wider than the vials themselves to extract for 1 hour.

While the samples in scintillation vials were being extracted, the filtrate containing centrifuge tubes were capped, vortexed vigorously for 1 minute and then centrifuged at 1380 g for 10 minutes to induce phase separation between chloroform and water. The tubes were removed from the centrifuge and the volumes of the two phases were recorded. Using a clean 9" Pasteur pipette, the bottom phase, which was the chloroform phase with dissolved lipids, was removed and transferred to a scintillation vial. Special attention was paid when removing the lipids as to not disturb the phases and cross contaminate. The remaining liquid, originally the upper aqueous phase, which contained the low molecular weight (LMW) compounds, was decanted into another scintillation vial. The scintillation vials containing the lipids and LMW compounds were set aside. Once an hour had elapsed, the TCA vials were removed from the heating block, which was then set to 60°C and after reaching 60°C, the vials containing the lipid and LMW compounds fractions were put in the heating block, uncapped, to dry.

After allowing the TCA vials to cool, the mixtures, including the filters, were transferred to the towers and filtered again to retain the TCA insoluble material on the new filter and catch the TCA soluble material below in the centrifuge tubes. A glass rod was used to draw out excess liquid from the filters. The TCA vials were rinsed with an additional 4 ml of room temperature 5% TCA and added to the tower. The liquid was again removed and with the vacuum on, the entire filter was removed to be placed back into the same TCA vial from which it came. These vials (i.e., the TCA insoluble fraction) contained the protein fraction and immediately received 4 ml of scintillation

cocktail. The filtrate (i.e., TCA soluble fraction) contained the polysaccharide-nucleic acid (poly-na) fraction and was placed in the heating block to evaporate solvents. Once the vials were dry, those containing the lipid fraction received 4 ml of scintillation cocktail, while the LMW compounds fraction and the poly-na fraction received 250 µl of Milli Q water to dissolve the fractions before receiving 4 ml of scintillation cocktail. All samples were counted in a Beckman™ LS-5000TD scintillation counter with a blank consisting of scintillation cocktail only under the ^{33}P protocol.

Chemical analysis: RNA biochemical fractionation

The RNA biochemical fractionation protocol was unlike the standard protocol in all facets. An overview of the RNA biochemical fractionation is presented in Fig. 10 resulting in four different fractions from the standard protocol. Copepods were rinsed with ASW-P and then placed into individual 500 µl RNAse free centrifuge tubes, to which 50 µl of TRI® reagent (Ambion, Inc.) was added to inactivate RNAses (Sambrook and Russell, 2001). The copepods were homogenized using the RNAse free pestles, which were rinsed with an additional 150 µl of TRI® reagent at completion of homogenization. The samples were then allowed to extract for 5 minutes at room temperature. After time elapsed, the samples were capped and placed in a –80°C freezer until analysis could be completed.

After thawing samples, 40 µl of 100% chloroform was added to each tube and vortexed vigorously for 25 seconds. The tubes were placed in the centrifuge to run at 10,000 g for 15 minutes at 4°C, which allowed for phase separation. The upper portion contained RNA and polysaccharides, while the interphase contained DNA and the lower organic phase contained proteins (Sambrook and Russell, 2001). At the end of the cycle,

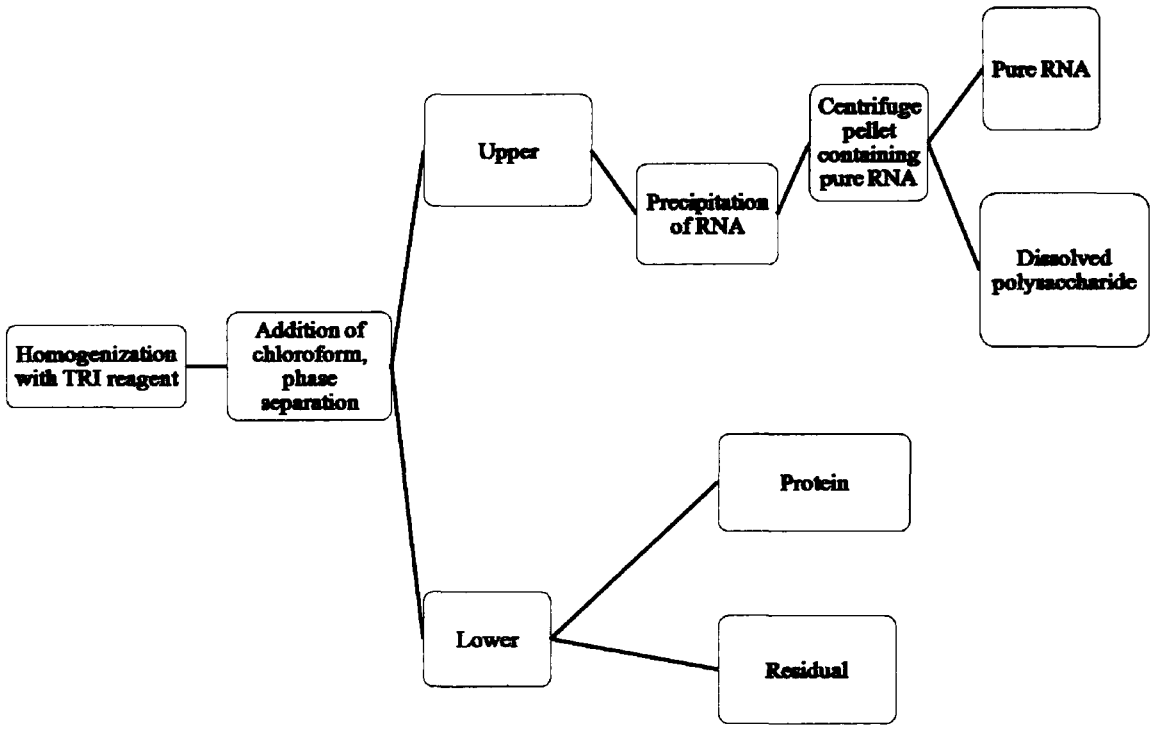

Fig. 10. Simplified overview of RNA biochemical fractionation process. The process was performed on individual copepods.

the upper 100 µl was taken using a 20-100 µl Ranin® pipette and deposited into a new 500 µl RNAse free centrifuge tube. When removing the RNA/polysaccharide layer, caution was used as to not disturb phases. The tubes containing the remaining interphase and bottom phase were placed in a refrigerator at 4°C until further processing.

To the tubes containing the RNA/polysaccharides phase, 50 µl of 100% isopropanol and 50 µl of precipitation reagent (Appendix IX) were added. The isopropanol selectively precipitates RNA from solution (Sambrook and Russell, 2001). The tubes were capped, vortexed at maximum speed for 10 seconds and then allowed to precipitate at room temperature for 10-15 minutes. The tubes were returned to the centrifuge to spin at 12,000 g and 4°C for 8 minutes. The liquid containing

polysaccharides was decanted into a scintillation vial. This was termed the polysaccharide fraction, although it may also have contained some unprecipitated RNA. The centrifuge tube was then gently touched upside down on a Kimwipe® to remove any remaining liquid clinging to the tube. To the RNA pellet, 200 µl of 75% ethanol was added and briefly vortexed. The centrifuge tubes were returned to the centrifuge, which was run at 12,000 x g for 8 minutes at 4°C. The liquid was again poured into the same scintillation vial the isopropanol (i.e., dissolved polysaccharides) was transferred into and the remaining steps were repeated. After these washes, and to dissolve the pellet, 200 µl of TE buffer was added to the centrifuge tube, which was subsequently capped and vortexed vigorously for 25 seconds. Finally, 100 µl of the RNA containing buffer was transferred into a scintillation vial, which was placed in a heating block kept between 40-60°C to dry-off solvents.

Tubes from the refrigerator containing the interphase and lower phase, which represented DNA and protein, respectively, were retrieved for processing. A 100 µl aliquot was removed from the lower phase (proteins) and placed into a scintillation vial. Any liquid remaining in the centrifuge tubes after this step, which would contain a mixture of DNA and protein, was decanted into another scintillation vial and was deemed the residual fraction. The polysaccharide, protein and residual scintillation vials were placed into a heating block with the RNA sample to evaporate liquids. Once samples were completely dry, 200 µl of Milli Q water was added to each vial to dissolve the sample. All dissolved samples received 4 ml of scintillation cocktail and the samples were read in the Beckman™ LS-5000TD scintillation counter as described previously.

Cell abundance

Cells were counted using Corning® polystyrene 24 well, 3.4 ml volume well plates and an Olympus™ CK2 inverted microscope. Aliquots of the samples were removed from their vials, placed into individual wells for counting, and artificial seawater was added to reduce the effect of evaporation. The well plates were left untouched for at least one hour to allow for settling of the algae on the plate. For each sample, all cells present in ten vertical and ten horizontal fields were counted with the magnification noted.

RESULTS

Dissolved phosphorus, particulate phosphorus and chlorophyll a content of Rhodomonas salina I

Dissolved phosphorus in the experimental media resulted in initially high values at T_0, followed by very low values for the duration of the time-series (Fig. 11). Initial modified f/2-Si, P-imbalanced (PI), and f/20-Si, P-balanced (PB), media contained 2.6 and 2.5 μM dissolved phosphorus (Fig. 11a), respectively. After 50 hours, the average uptake for *Rhodomonas salina* cells was 0.49 fmol/cell/hr for PI and 0.79 fmol/cell/hr for PB algae (Fig. 11b).

Cell abundance (Fig. 12a) was significantly different (ANOVA, df=23, F=6.47, p-value=0.019) between treatments with the PI *R. salina* containing 1.5 times more cells (Table 2). There were also significant differences in cell-specific particulate phosphorus and Chl *a* between the PB and PI (Table 2; Figs. 12b and 12c). The PB treatment contained 1.8 times more phosphorus per cell and there was a 4.6 fold difference in cell specific Chl *a* values between PI and PB cultured *R. salina* (Table 2). The algae were introduced to the copepods at ~115 hours as indicated by arrows. Note that the Chl *a* time series only contains samples from 140 hours and on because earlier measurements were unusable due to calibration problems with the spectrophotometer. The difference in Chl *a* concentrations described above was also visibly apparent with PI cultured *R. salina* appearing darker (i.e., more pigment) than the PB cultured *R. salina* (Fig. 13). This pigment variation was also an early indication that the algae had, in fact, been biochemically altered, which was further substantiated by the results of the biochemical fractionation that follows.

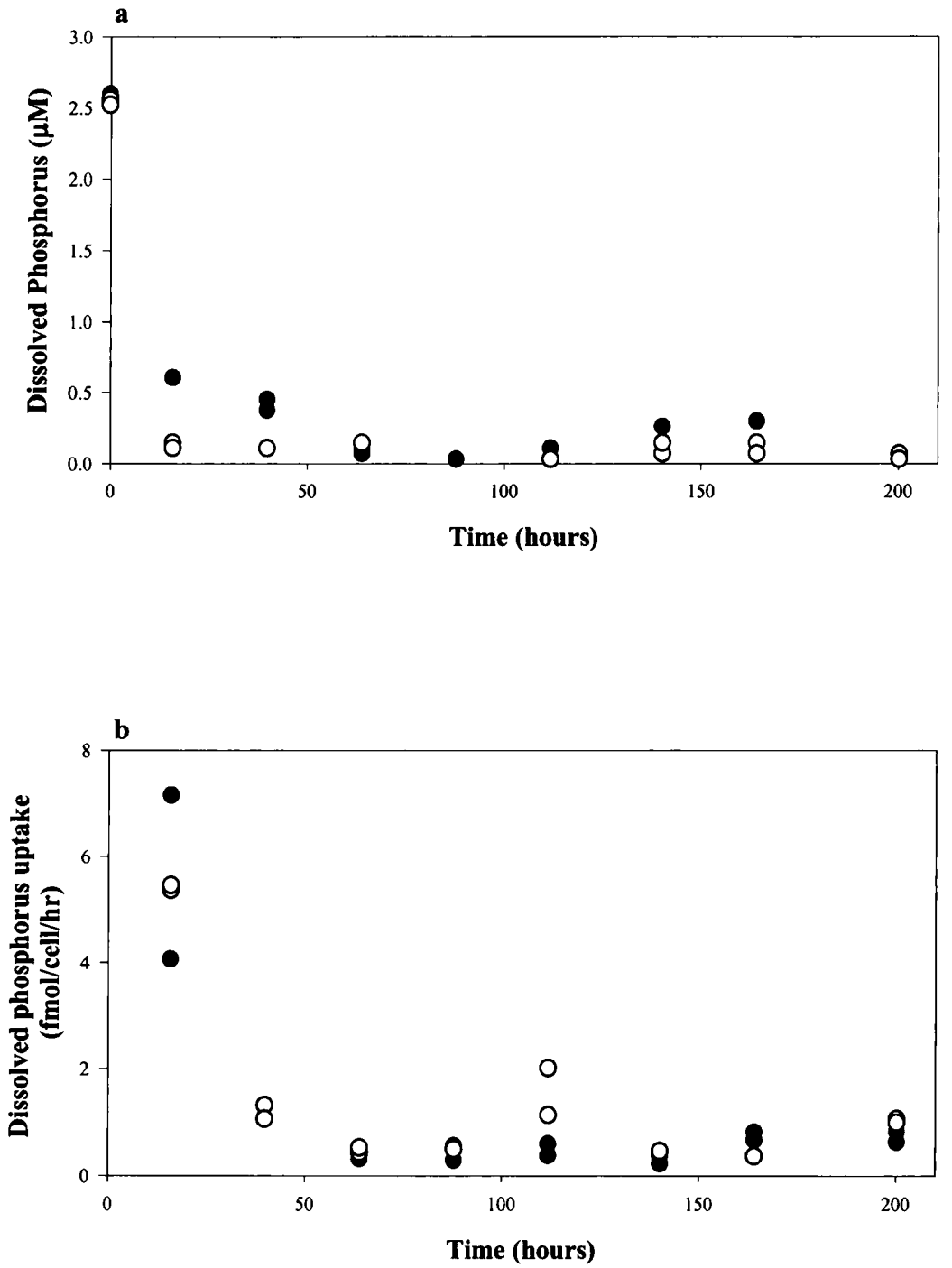

Fig. 11. Concentration of dissolved phosphorus in the P-imbalanced (PI) and P-balanced (PB) media before and during the ^{33}P incorporation experiment time-series (a) and *R. salina* cell specific phosphorus uptake (b). PI and PB cultured *R. salina* are presented as filled and open circles, respectively. Dissolved phosphorus sample volume was 20 ml.

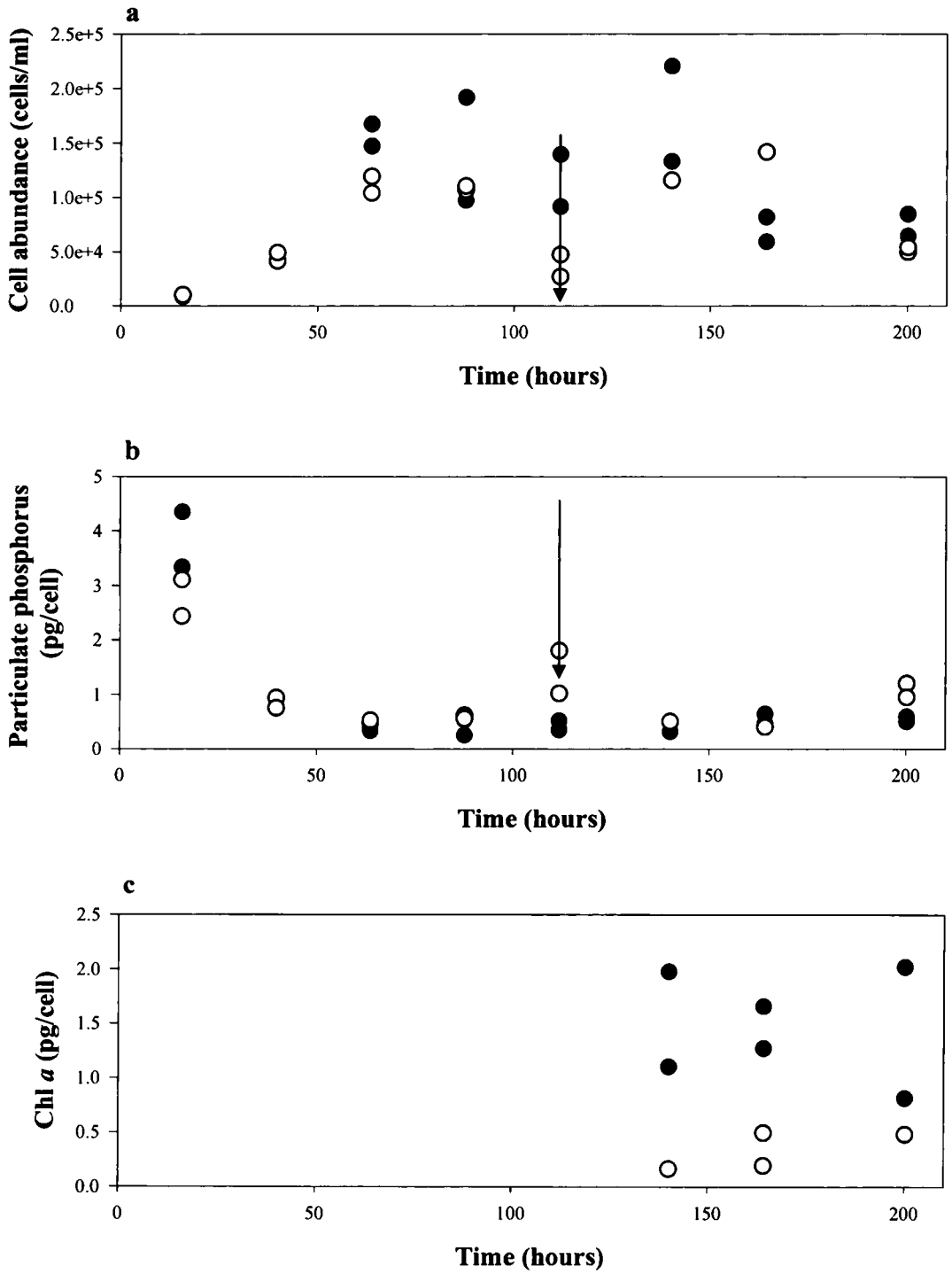

Fig. 12. Cell abundance of *R. salina* and cell specific values of particulate phosphorus and Chl *a* during the ^{33}P incorporation experiment time-series. P-imbalanced (PI) and P-balanced (PB) cultured *R. salina* are presented as filled and open circles, respectively. Arrows at ~115 hours indicate when *A. tonsa* began feeding on the experimental *R. salina*. Panels are cell abundance (a), particulate phosphorus (b) and Chl *a* (c). Sample volumes were 25 ml for particulate phosphorus and 5 ml for Chl *a*.

Table 2
Means and standard deviations for cell abundance, cell specific values of phosphorus, and Chl *a* during the ^{33}P incorporation experiment time-series after 42 hours for *R. salina* cultured on P-imbalanced (PI) and P-balanced (PB) media. Results of pair-wise (between treatments), one-way ANOVAs performed on each measurement are given in the last column. Those ANOVAs with p-values <0.05 are in bold.

	PI treatment			PB treatment			ANOVA	
	n	Mean	Standard deviation	n	Mean	Standard deviation	F	p-value
Cell abundance (cells/ml)	12	122418	51755	12	79809	39133	6.47	**0.019**
Particulate phosphorus (pg/cell)	12	0.44	0.12	12	0.80	0.40	10.40	**0.004**
Chl *a* (pg/cell)	6	1.4	0.48	4	0.3	0.15	16.80	**0.005**

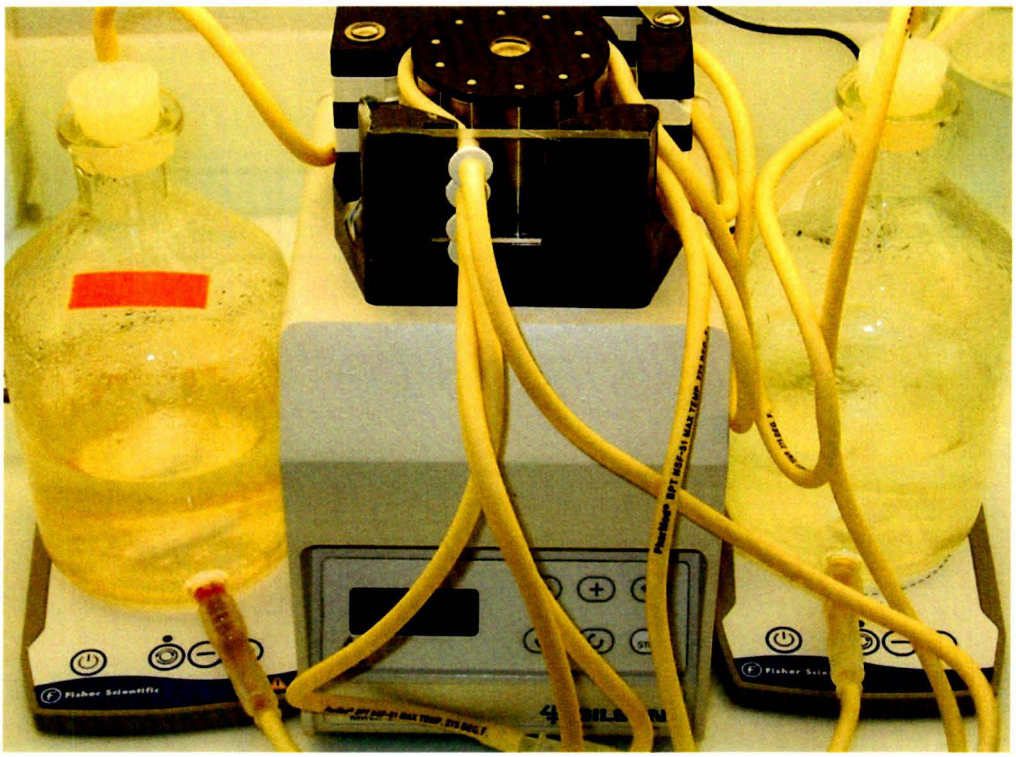

Fig. 13. Algae cultures during incorporation experiments (at 167 hours) with P-imbalanced (PI) culture on the left and P-balanced (PB) culture on the right. PI cultures appeared much darker than the PB cultures because the PI culture contained greater concentration of cells and more Chl *a* per cell than the PB culture (Table 2).

^{33}P uptake and allocation in Rhodomonas salina

In both the P-imbalanced (PI) and P-balanced (PB) treatment *Rhodomonas salina* cultures, the first time-point (~16 hours) exhibited high radioactive counts (dpm), while subsequent values were lower (Figs. 14a, b, d and e). Thus, the average cell specific radioactivity was calculated after 16 hours. The fractions leveled off after 16 hours in all fractions, which in most instances was well before the copepods were presented with the algae, as indicated by arrows around 115 hours (Fig. 14), with the exception of one time-point, likely due to the severe drop in cell abundance (Fig. 12a). No biochemical fraction or the total radioactivity followed the anticipated Michaelis-Menten uptake kinetics (Fig. 1a). *R. salina* incorporated more ^{33}P in all fractions of the PB treatment than in the PI treatment, except for the LMW compounds (Table 3). In the protein, lipid, and polysaccharide-nucleic acid (poly-na) fractions and in the totals, average values were 1.9, 3.3, 1.7 and 1.9 times greater, respectively in the PB cultured *R. salina* (Table 3). PB cultured *R. salina* incorporated more ^{33}P than PI cultured *R. salina*, and both treatments demonstrated the largest incorporation in the protein and poly-na fractions (Table 3).

One-way ANOVAs, summarized in Table 3, verified the graphical differences in cell specific *R. salina* ^{33}P values in the protein, lipid, poly-na fractions and in the totals. The differential allocation of ^{33}P therefore reflected biochemical changes in the algae. The most important objective was thus achieved: to obtain biochemically altered algae for the copepods to feed on, solely based on phosphorus concentration relative to other nutrients, while the absolute phosphorus concentration remained the same in both treatments.

To determine the degree of homeostasis in *R. salina*, the cellular percent ^{33}P of

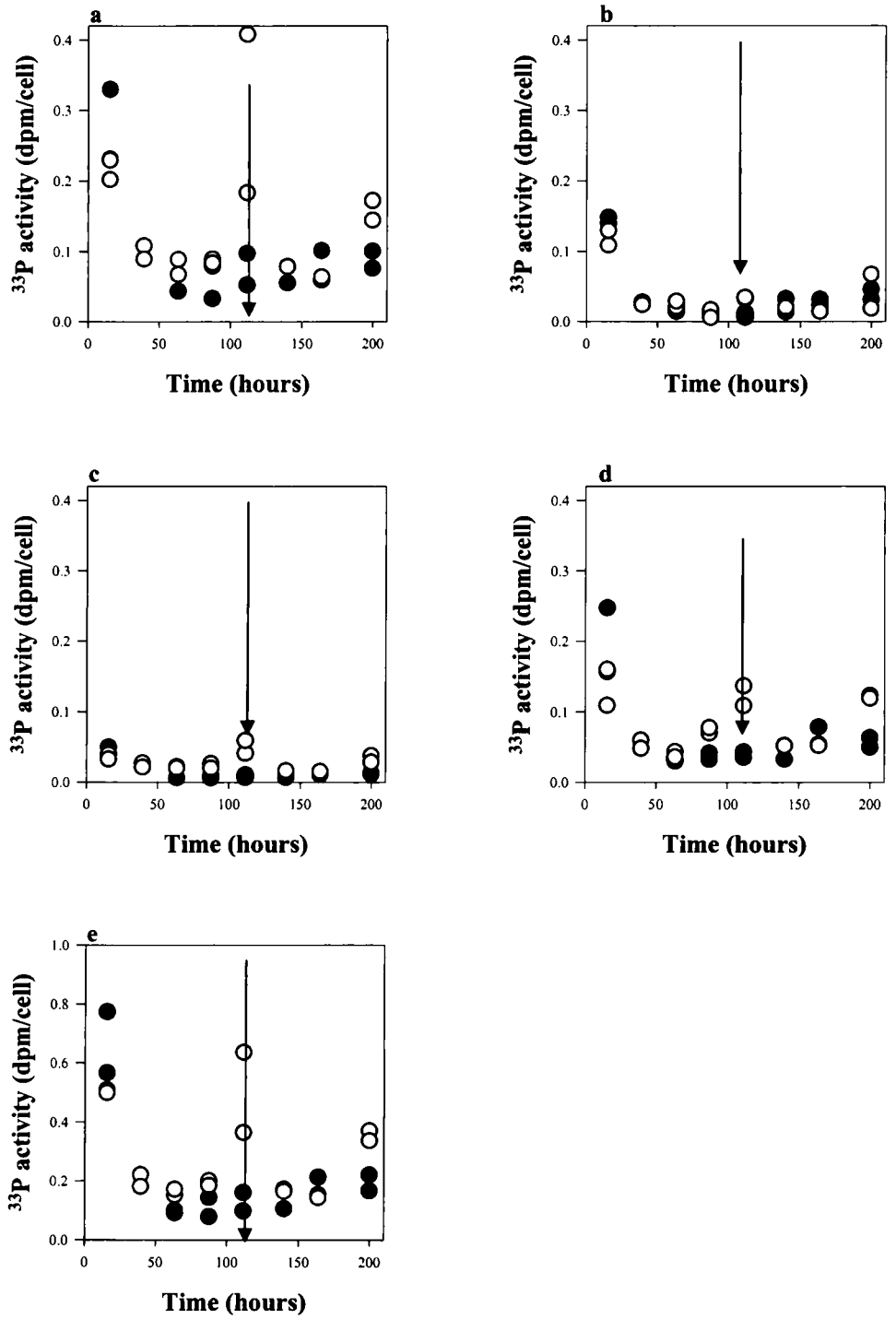

Fig. 14. Cellular ^{33}P activity in biochemical fractions of *R. salina*, as well as in the totals (sum of all fractions) during the ^{33}P incorporation experiment time-series. Panels are (left to right) protein (a), LMW compounds (b), lipid (c), poly-na (d) and totals (e) with filled circles indicating P-imbalanced (PI) and open circles indicating P-balanced (PB) cultured *R. salina*. Arrows at ~115 hours indicate time when *A. tonsa* began receiving the experimental *R. salina*. Sample volume was 20-25 ml. Duplicate samples were taken for each treatment at every time-point.

Table 3
Means and standard deviations for cellular ^{33}P radioactivity (dpm) in biochemical fractions (protein, LMW compounds, lipid, and poly-na) in *R. salina* at steady state (42+ hours). Results of pair-wise (between treatments), one-way ANOVAs are given in the last column. Those ANOVAs with p-values <0.05 are in bold.

	PI treatment			PB treatment			ANOVA	
Biochemical Fraction	**n**	**Mean**	**Standard deviation**	**n**	**Mean**	**Standard deviation**	**F**	**p-value**
Protein	12	0.067	0.024	12	0.130	0.096	5.77	**0.026**
LMW compounds	12	0.020	0.012	12	0.025	0.015	1.93	0.180
Lipid	12	0.008	0.003	12	0.027	0.013	27.02	**<0.001**
Poly-na	12	0.045	0.015	12	0.076	0.035	16.49	**0.001**
Total	12	0.140	0.048	12	0.260	0.140	10.00	**0.005**

total signal in each fraction was examined. The percentages are presented stacked for each treatment and at each time-point in Fig. 15. The protein fraction contained the greatest percentage ^{33}P, followed by poly-na, LMW compounds and lipid fractions (Fig. 15; Table 4). Both PI and PB cultured *R. salina* incorporated roughly the same percent ^{33}P into all fractions, except in the lipid fraction (Fig. 15; Table 4), which contained approximately 2 times more ^{33}P in the PB treatment algae (one-way ANOVA; df=23, F=113.84, p<0.001). Thus, while differences in cellular radioactivity occurred in three out of four fractions, only one fraction, based on percent ^{33}P incorporation, was different.

^{33}P uptake and biochemical allocation in Acartia tonsa I

The incorporation of ^{33}P into *Acartia tonsa* was vastly different between P-imbalanced (PI) and P-balanced (PB) treatments (Fig. 16). All incorporation patterns followed a linear trend with no indication of Michaelis-Menten saturation (Fig. 1a). Linear models were therefore fit to the data and the slopes were compared between treatments and among biochemical fractions using ANCOVAs. A summary of the tests of homogeneity of slopes is shown in Table 5. There was a quicker and overall greater

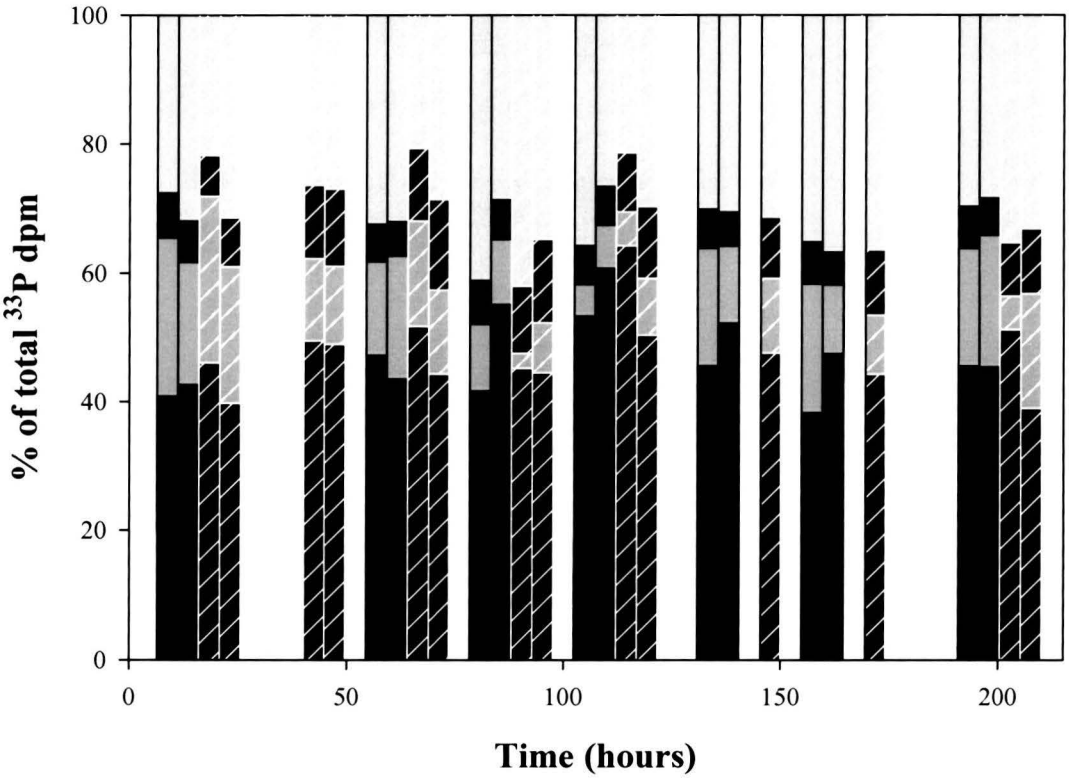

Fig. 15. Cellular percentages of ^{33}P in the biochemical fractions during the ^{33}P incorporation experiment time-series. The fractions are (from bottom to top): protein, LMW compounds, lipid and poly-na. Solid bars represent P-imbalanced (PI), while hatched lines represent P-balanced (PB) *R. salina*.

Table 4
Means and standard deviations for cellular percentages of ^{33}P in the biochemical fractions of *R. salina* during the ^{33}P incorporation experiment time-series. Results of pair-wise (between treatments), one-way ANOVAs are given in the last column. Those ANOVAs with p-values <0.05 are in bold.

	PI treatment			PB treatment			ANOVA	
Biochemical Fraction	**n**	**Mean**	**Standard deviation**	**n**	**Mean**	**Standard deviation**	**F**	**p-value**
Protein	12	48.01	6.28	12	48.39	6.21	<0.01	0.980
LMW compounds	12	13.83	5.40	12	10.23	4.63	2.49	0.130
Lipid	12	5.87	0.53	12	10.83	1.63	113.84	**<0.001**
Poly-na	12	32.33	4.22	12	30.56	6.25	0.38	0.540

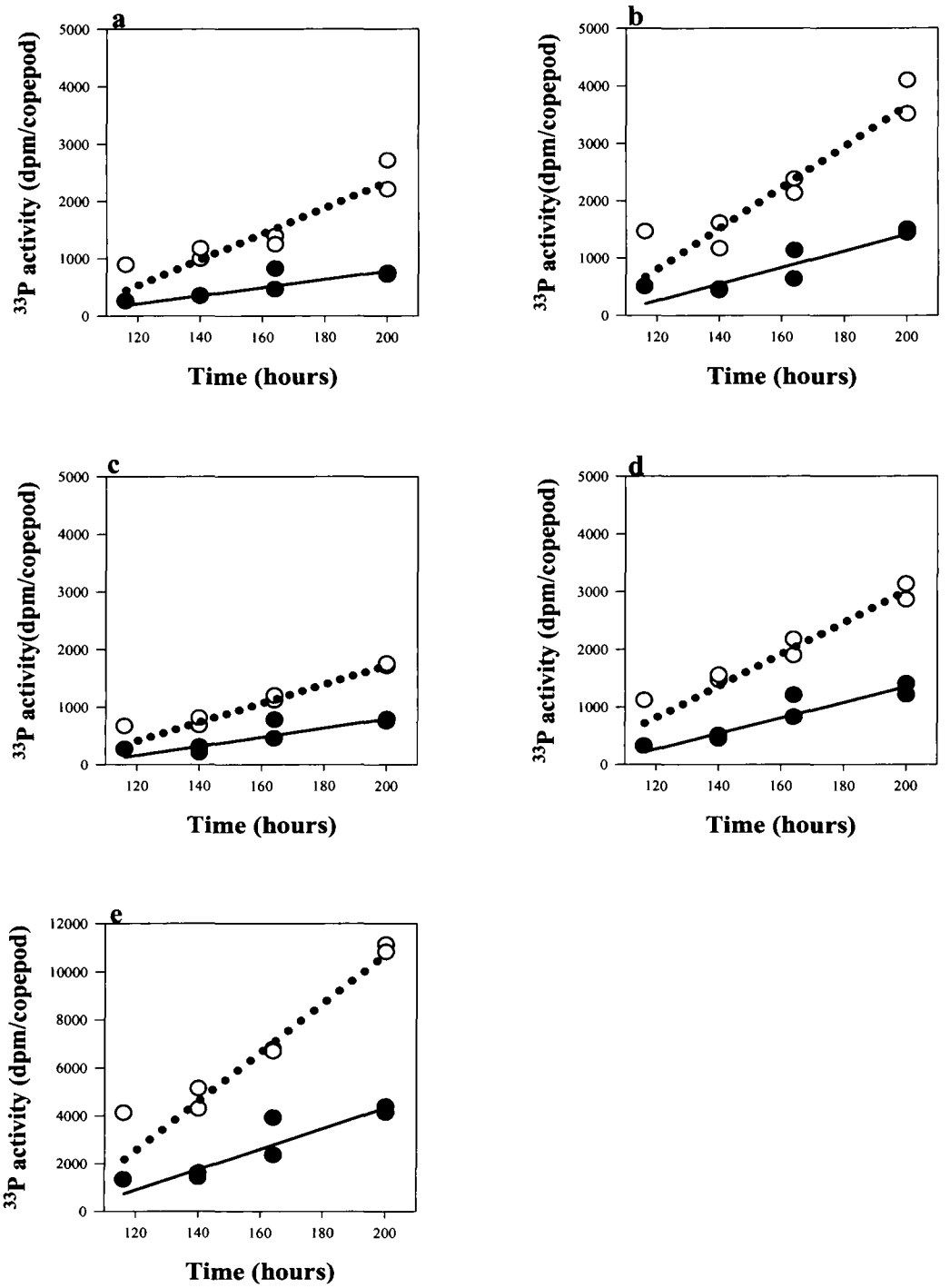

Fig. 16. ^{33}P activity in biochemical fractions and in the totals (sum of all fractions) of *A. tonsa* fed P-imbalanced and P-balanced *R. salina* during the ^{33}P incorporation experiment time-series. Panels are (left to right) protein (a), LMW compounds (b), lipid (c), poly-na (d) and totals (e) with filled circles indicating P-imbalanced (PI) and open circles indicating P-balanced (PB) fed *A. tonsa*. Slopes of incorporation (PI: solid line; PB: dotted line) were significantly different between all fractions and in the totals, according to a test of homogeneity of slopes using the ANCOVA procedure (Table 5). Copepod samples consisted of 4-10 individuals per filter sample.

^{33}P incorporation into the PB treatment *A. tonsa* (Fig. 16; Table 5), with 2.2-3 times more ^{33}P dpm incorporated into the various fractions and totals (Fig. 16; Table 5). Additionally, Fig. 17 illustrates the range of radioactivity within each treatment. The lowest level of radioactivity in the PB fed copepods was in the lipid fraction (Fig. 17b), and was greater than the highest level (LMW compounds) of the PI fed copepods (Fig. 17a). As such, all fractions and the totals were significantly different between the two treatments (Fig. 16; Table 5). Within the PB fed *A. tonsa*, the LMW compounds fraction had the greatest incorporation rate followed by the poly-na, protein and lipid fractions (Table 5). Incorporation by PI fed *A. tonsa* ranking was slightly different: LMW compounds, poly-na, lipid and protein (Table 5), in descending order. In each treatment, equivalent amounts of stable (^{31}P) and radioactive phosphorus (^{33}P) were available for *Rhodomonas salina*, but the differences in media appear to have produced differences in algal radioactivity (Figs. 14a,c-e; Table 3) that was further amplified by *A. tonsa* (Fig. 16; Table 5).

Table 5
Time-series ^{33}P incorporation rates into biochemical fractions of *A. tonsa* fed P-imbalanced (PI) or P-balanced (PB) *R. salina* during the incorporation experiment. These incorporation rates represent the slopes of the linear regressions shown in Figs. 16 and 17. Results of pair-wise (between treatments), one-way ANOVAs are given in the last column. Those ANOVAs with p-values <0.05 are shown in bold.

	^{33}P incorporation rate (dpm/copepod/hr)		**ANCOVA**	
Biochemical fraction	**PI treatment**	**PB treatment**	**F**	**p-value**
Protein	7.10	22.65	40.34	**0.002**
LMW compounds	14.39	35.74	49.20	**0.003**
Lipid	8.00	16.47	46.07	**0.008**
Poly-na	13.56	27.36	60.57	**0.005**
Total	43.04	102.22	63.77	**0.001**

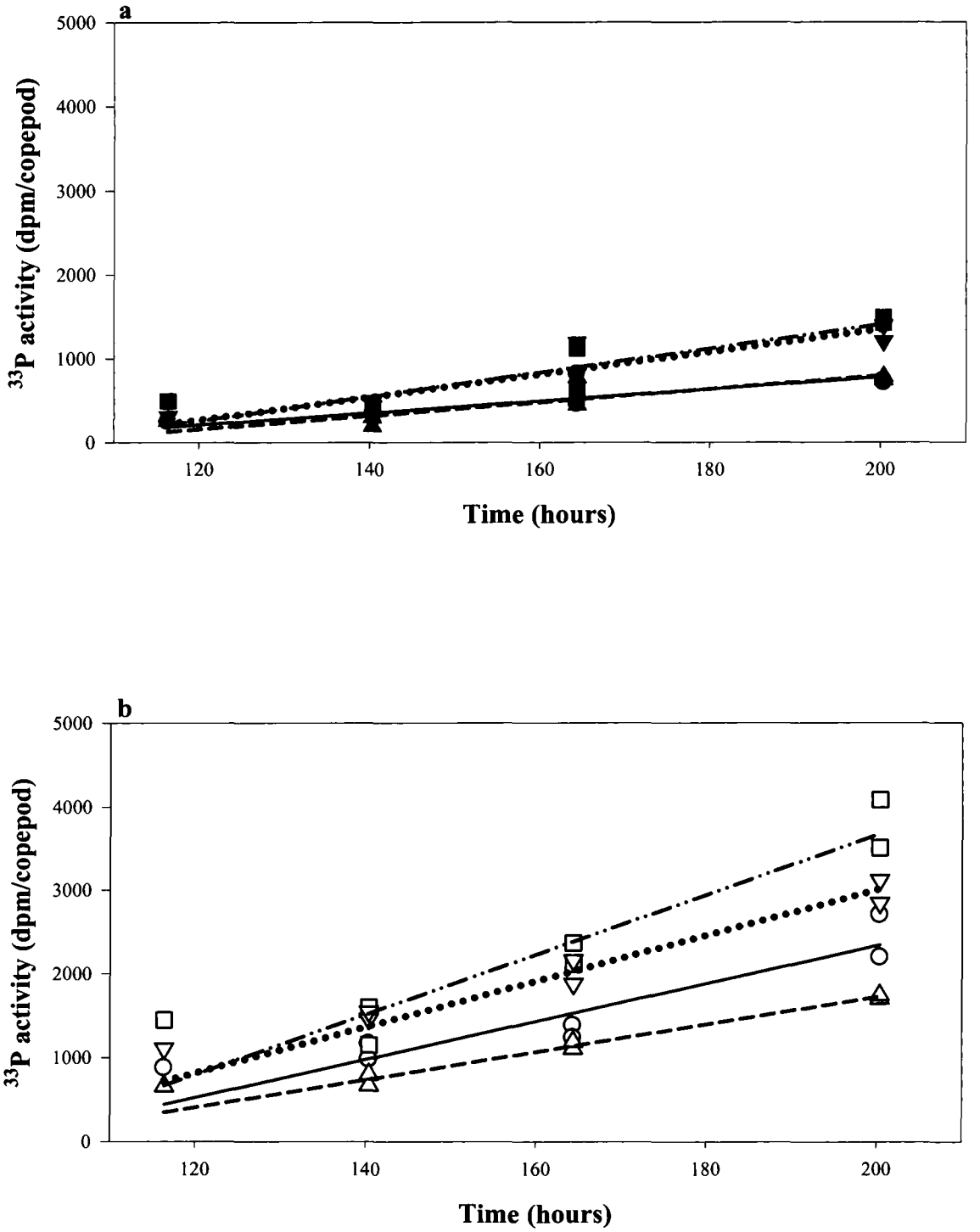

Fig. 17. ^{33}P activity in biochemical fractions of *A. tonsa* fed P-imbalanced (a) and P-balanced (b) *R. salina* during the ^{33}P incorporation experiment time-series. Symbols are CIRCLE (protein), SQUARE (LMW compounds), TRIANGLE UP (lipid) and TRIANGLE DOWN (poly-na). Slopes of incorporation (protein (------), LMW compounds (—..—), lipid (. . . . . .) and poly-na (- - - - -)) had varying levels of significance between fractions within each treatment, according to a test of homogeneity of slopes using the ANCOVA procedure (Table 6). Copepod samples consisted of 4-10 individuals per filter sample.

Given differences between treatments in the biochemical fractions and in their totals, fractions by treatment were examined as pair-wise ANCOVAs to determine variation in fractional incorporation rate. The differences among biochemical fractions (i.e., Protein v LMW compounds) were more complex than in the treatments (i.e., PI v PB). Most notably, the LMW compounds and poly-na fractions incorporated ^{33}P more rapidly than the protein and lipid fractions (Tables 5-6). All fractional sets that were significantly different contained either LMW compounds or poly-na (Table 6) with the PI fed *A. tonsa* demonstrating more differences between fractions than the PB fed *A. tonsa*.

Table 6
Summary results of pair-wise (between fractions) ANCOVAS comparing the slopes of ^{33}P incorporation into biochemical fractions within *A. tonsa* fed either P-imbalanced (PI) or P-balanced (PB) *R. salina*. Those ANCOVAs with p-values < 0.05 are in bold. The slopes of fractions compared are listed in Table 5.

	PI treatment			PB treatment		
Biochemical fractional sets	**df**	**F**	**p-value**	**df**	**F**	**p-value**
Protein v LMW compounds	15	6.60	**0.025**	15	4.33	0.060
Protein v Lipid	15	0.16	0.700	15	2.16	0.170
Protein v Poly-na	15	6.70	**0.024**	15	0.86	0.370
LMW compounds v Lipid	15	5.06	**0.044**	15	11.98	**0.005**
LMW compounds v Poly-na	15	0.08	0.790	15	1.79	0.210
Lipid v Poly-na	15	4.95	**0.046**	15	6.85	**0.022**

Homeostasis was also investigated in *A. tonsa* by examining how the percent ^{33}P incorporated fluctuated in the fractions, as a percent of total ^{33}P, during the time-series and between treatments. In both PI and PB treatments, the LMW compounds and poly-na fractions comprised the greatest percentages of ^{33}P, while the protein and lipid fractions contained the least (Table 7; Fig. 18). The average percent ^{33}P incorporation in each fraction was not significantly different between treatments (Table 7). Thus, the

differences in incorporation rate (Table 5) had no effect on percent incorporation (Table 7), *A. tonsa* merely incorporated ^{33}P proportionally into its tissues regardless of diet.

Table 7
Means and standard deviations for percent ^{33}P incorporation in biochemical fractionations of *A. tonsa* during the incorporation experiment. Results of pair-wise (between treatment), one-way ANOVAs are given in the last column. No fraction was statistically significant between treatments as no p-value was <0.05.

	PI treatment			PB treatment			ANOVA	
Biochemical fraction	**n**	**Mean**	**Standard deviation**	**n**	**Mean**	**Standard deviation**	**F**	**p-value**
Protein	8	19.46	2.56	8	20.93	2.67	1.26	0.28
LMW compounds	8	31.98	3.89	8	34.45	5.55	1.07	0.32
Lipid	8	16.54	5.01	8	15.03	3.34	0.50	0.49
Poly-na	8	32.02	5.58	8	29.59	2.66	1.24	0.28

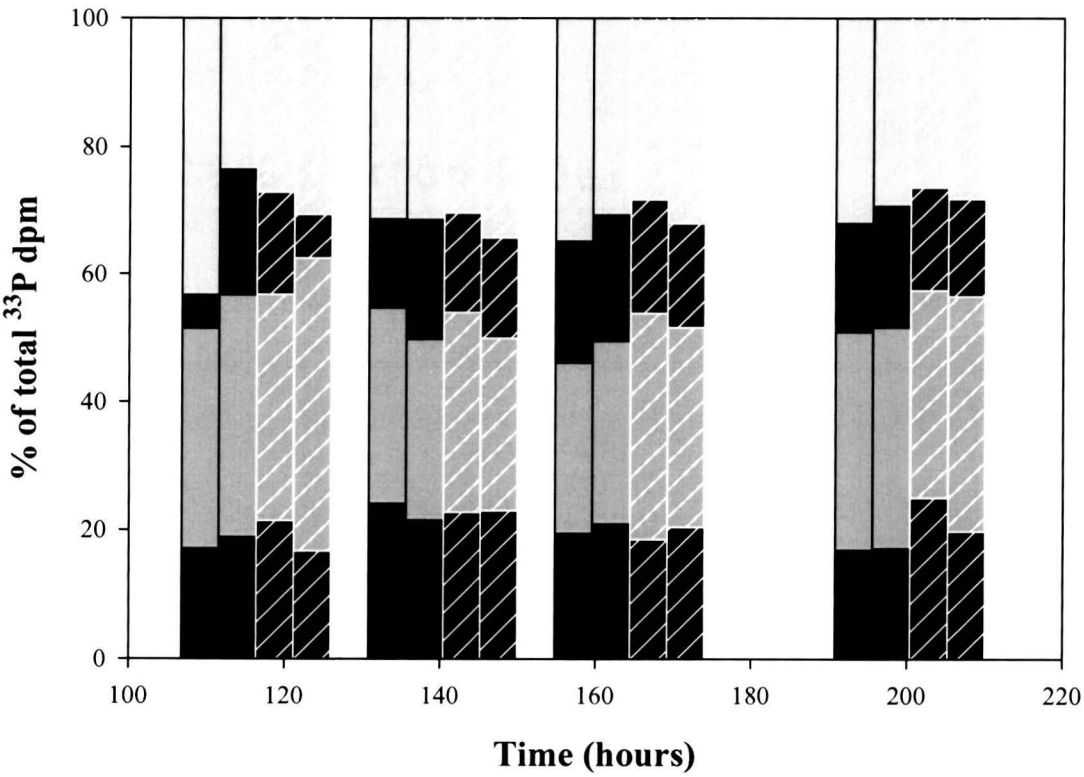

Fig. 18. Percentages of ^{33}P in the biochemical fractions of *A. tonsa* during the ^{33}P incorporation experiment time-series. The fractions are (from bottom to top): protein, LMW compounds, lipid and poly-na. Solid bars represent P-imbalanced (PI), while hatched lines represent P-balanced (PB) fed *A. tonsa*. Duplicate samples were taken for each treatment at every time-point.

Examining the relationships between the algae and copepod percent ^{33}P incorporation revealed ambiguous results. When the two were compared, significant differences occurred in the protein, LMW compounds and lipid fractions of the PI and PB treatments (Figs. 15 and 18; Table 8). Additionally, in both the PI and PB treatments, *R. salina* had greater ^{33}P percent incorporation in the protein fraction, while *A. tonsa* incorporated proportionally more into the LMW and lipid fractions (Tables 4, 7and 8). Thus, while each organism regulated the incorporated nutrients during their time-series, their allocation was different in all fractions, except poly-na.

Table 8
Summary results for pair-wise (between organism), one-way ANOVAs for percent ^{33}P incorporation in *R. salina* and *A. tonsa* based on values from Figs. 15 and 18. Those ANOVAs with p-values <0.05 are in bold.

	PI treatment			PB treatment		
Biochemical fraction	**df**	**F**	**p-value**	**df**	**F**	**p-value**
Protein	19	128.42	**<0.001**	19	91.79	**<0.001**
LMW compounds	19	68.45	**<0.001**	19	91.89	**<0.001**
Lipid	19	63.78	**<0.001**	19	19.46	**<0.001**

Dissolved phosphorus, particulate carbon, particulate nitrogen, particulate phosphorus and Chlorophyll a content of Rhodomonas salina II

Dissolved phosphorus concentrations in the medium were similar, often nearly identical, between PI and PB treatments (Fig. 19a). Initial concentrations at T_0 were high for both treatments (2.4 μM for PI and 2.7 μM for PB), followed by a sharp decrease to 0.04 μM (Fig. 19a) for both treatments. Therefore, the difference between initial concentrations and those at each time-point resulted in a 2+ μM uptake by the algal cultures per day. The phosphorus uptake (from 42 hours on) for *Rhodomonas salina* cells

was 1.03 fmol P/cell/hr in the PI treatment medium and 0.63 fmol P/cell/hr in the PB treatment medium (Fig. 19b).

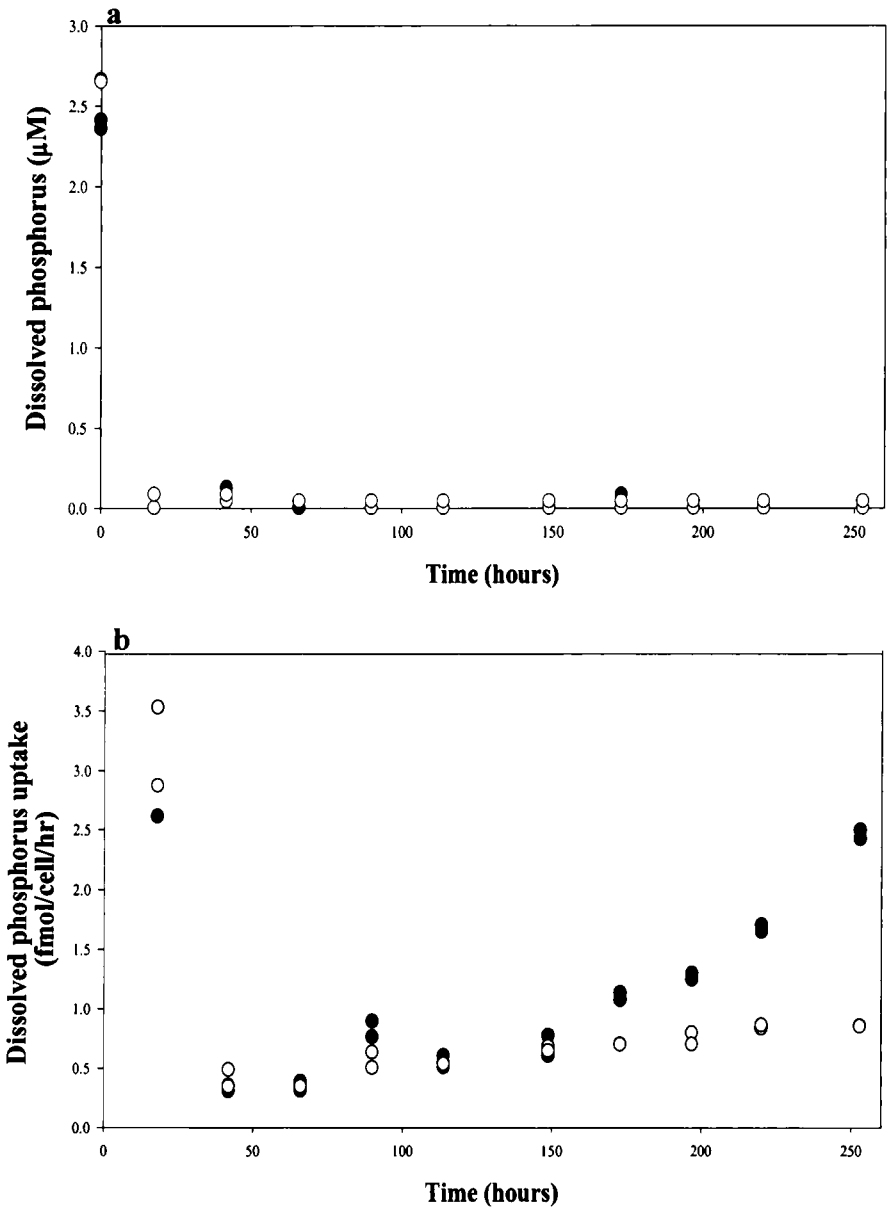

Fig. 19. Concentration of dissolved phosphorus in the P-imbalanced (PI) and P-balanced (PB) media before and during the ^{33}P incorporation experiment time-series (a) and *R. salina* cell specific phosphorus uptake (b). PI and PB cultured *R. salina* are presented as filled and open circles, respectively. Dissolved phosphorus sample volumes were 20 ml.

The cell abundance and chemical analysis of *R. salina* for the second copepod incorporation experiment produced variable results. Cell abundance demonstrated an initial increase during the first 48 hours, followed by a continual decrease to much lower values by the end of the experiment (Fig. 20d). The cell specific values for particulate carbon and Chl *a* started with high values, which declined to lower values after 50 hours, where they remained for the duration of the experiment (Figs. 20a-b). On the other hand, particulate nitrogen and phosphorus decreased to lower values by 42 hours (Figs. 20c and 20e). All chemical components appeared to reach steady state, with slight variability, before the copepods were added at 131.5 hours, as indicated by arrows (Figs. 20a-c and 20e). When only the steady state values were taken into consideration, significant differences occurred in all metrics but the cell specific phosphorus (Figs. 20a-c and 20e; Table 9). The PI *R. salina* contained 3.5 times more Chl *a*, 1.7 times more particulate nitrogen, and 1.2 times more particulate carbon per cell than the PB *R. salina*, while the PB *R. salina* contained 1.5 times more cells (Figs. 20a-c and e; Table 9). Based on mean values for carbon and cell abundance in Table 9, carbon per liter was 4,538 and 5,488 μg for PI and PB, respectively, verifying that the copepods were not food limited.

The Redfield ratio is defined as 106:16:1 (molar C: N: P), resulting in a C:N of 6.7, C:P of 106 and N:P of 16. The cellular C:N, C:P and N:P molar ratios are presented in Figure 21. The average (90+ hours) cellular molar C: N: P ratio for *R. salina* was 381:61:1 and 371:42:1 for PI and PB treatments (Table 10), respectively. As a consequence, the ratios were much higher than Redfield in almost all comparisons of the PI cultured *R. salina* (C:N=6.3; C:P=381; N:P=61) and all comparisons of the PB cultured *R. salina* (C:N=8.8; C:P=371; N:P=42). C:N and N:P ratios were significantly

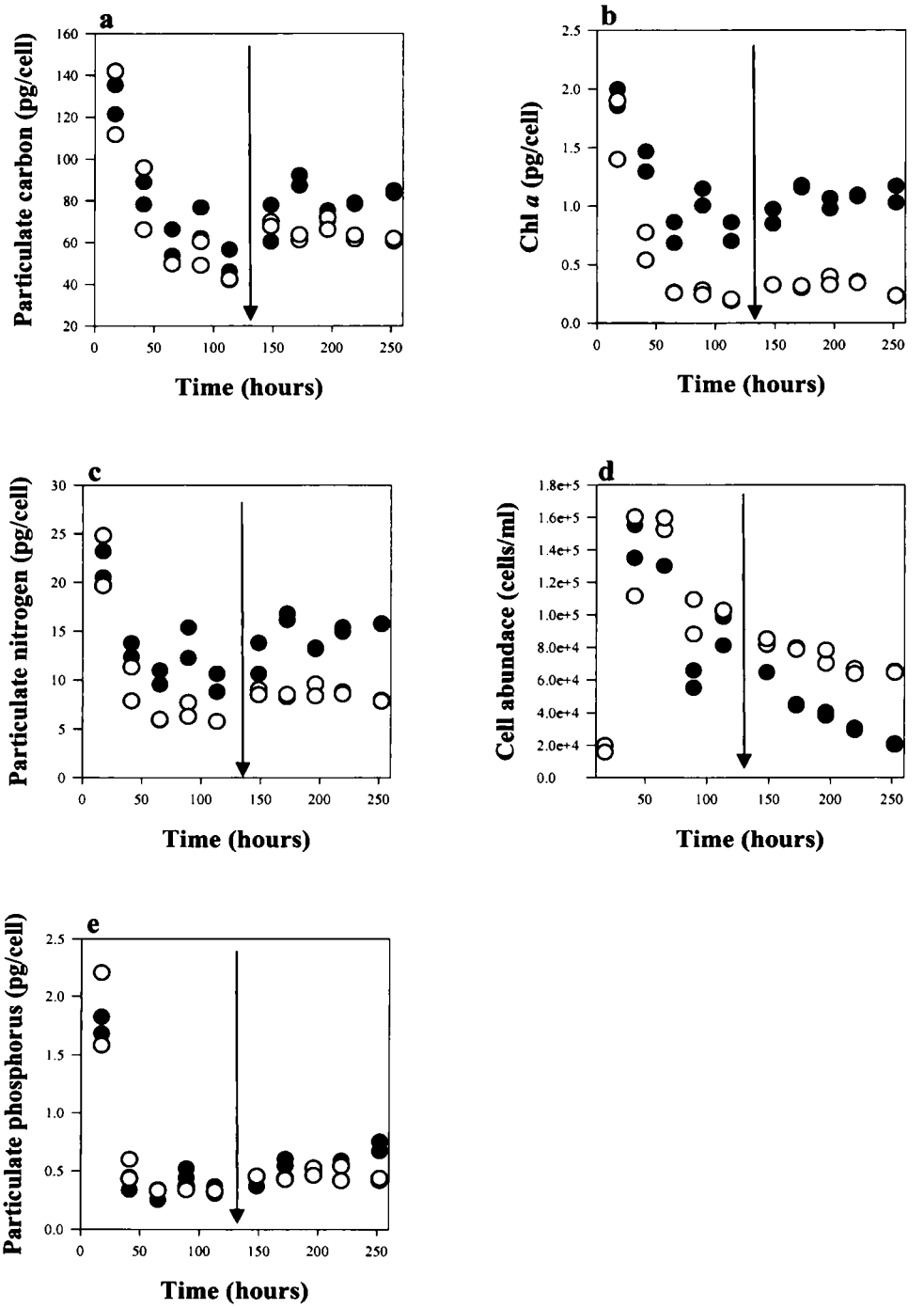

Fig. 20. Cell specific values of particulate carbon, nitrogen and phosphorus; Chl *a* and cell abundance of *R. salina* before and during the ^{33}P incorporation experiment time-series. P-imbalanced (PI) and P-balanced (PB) cultured *R. salina* are presented as filled and open circles, respectively. Arrows at ~131 hours indicate when *A. tonsa* began feeding on experimental *R. salina*. Panels are particulate phosphorus (a), Chl *a* (b), particulate nitrogen (c), cell abundance (d) and particulate phosphorus (e). Sample volumes were 25 ml for particulate carbon, particulate nitrogen and particulate phosphorus, whereas Chl *a* samples were 5 ml.

different between the PI and PB *R. salina* cultures (Figs. 21a and 21c; Table 10).

Conversely, the C:P ratio was similar in the two cultures (Fig. 21b; Table 10).

Table 9
Means and standard deviations for cell abundance (66+ hours), cell specific particulate carbon (42+ hours), nitrogen (42+ hours) and phosphorus (42+ hours); and Chl *a* (66+ hours) during the ^{33}P incorporation experiment for *R. salina* cultured on P-imbalanced (PI) and P-balanced (PB) media. Results of pair-wise (between treatment), one-way ANOVAs performed on each measurement are given in the last column. Those ANOVAs with p-values <0.05 are in bold.

Chemical components	PI treatment			PB treatment			ANOVA	
	n	Mean	Standard deviation	n	Mean	Standard deviation	F	p-value
Cell abundance (cells/ml)	16	62,304	39,416	16	90,047	29,151	5.12	**0.031**
Carbon (pg/cell)	18	72.83	13.10	18	60.95	12.39	7.95	**0.008**
Nitrogen (pg/cell)	18	13.22	2.42	18	7.81	1.48	78.74	**<0.001**
Phosphorus (pg/cell)	18	0.54	0.32	18	0.52	0.43	0.03	0.869
Chl *a* (pg/cell)	16	1.0	0.16	16	0.3	0.06	343.64	**<0.001**

Table 10
Means and standard deviation for cellular C:N, C:P and N:P molar ratios for P-imbalanced (PI) and P-balanced (PB) *R. salina* from 90+ hours. Results of pair-wise (between treatment), one-way ANOVAs performed on each measurement are given in the last column. Those ANOVAs with p-values <0.05 are in bold.

Cellular molar ratios	PI treatment			PB treatment			ANOVA	
	n	Mean	Standard deviation	n	Mean	Standard deviation	F	p-value
C:N	14	6.25	0.25	14	8.88	0.33	540.60	**<0.001**
C:P	14	381.01	41.71	14	371.24	30.91	0.60	0.450
N:P	14	61.00	6.10	14	41.79	3.19	148.01	**<0.001**

^{33}P uptake and allocation in Acartia tonsa II

Since individual copepods were analyzed in this second round of experiments, ^{33}P

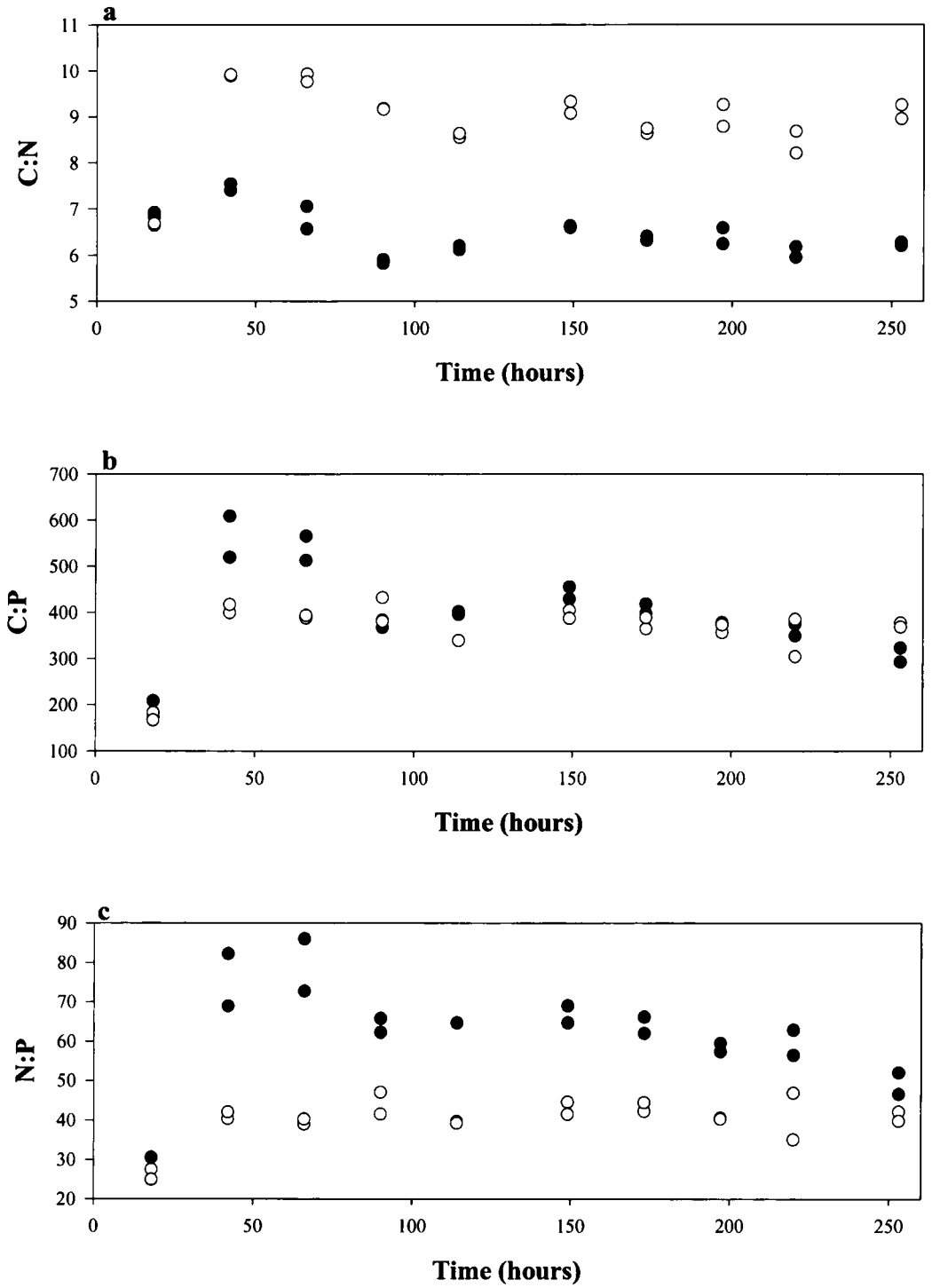

Fig. 21. Time-series cell specific molar ratios for P-imbalanced (PI) and P-balanced (PB) cultured *R. salina*. PI and PB *R. salina* are presented as filled and open circles, respectively. Panels are C:N (a), C:P (b) and N:P (c).

dpm variability in each fraction and at each time-point was higher than in previous experiments (Fig. 22). In most cases, the copepods continued to exhibit linear ^{33}P uptake. The biochemical fractions of interest, for this part of the study, were primarily protein and RNA, while the polysaccharide and residual (interphase between protein and RNA/polysaccharide) fractions, were secondary and not as precisely delineated. Overall, the greatest radioactivity was incorporated into the polysaccharide and residual fractions (Figs. 22c-d), while the lowest dpm was found in the RNA and protein fractions (Figs. 22a-b). The RNA fraction saturated rapidly in both treatments during the time course. The K_m values, or time to 50% saturation, were only 16.3 and 11.7 hours for PI and PB fed *Acartia tonsa*, respectively. This resulted in complete saturation of the RNA fraction in both treatments before 170 hours, which was <48 hours from the start of the experiment (Fig. 22a). The protein fraction in the PB fed copepods, on the other hand, had a K_m value of 50.6 hours and consequently became saturated much later (~ 230 hours) (Fig. 22b). The protein fraction of the PI treatment did not saturate (Fig. 22b) and neither did any treatment in the other two fractions (Figs. 22c-d).

As the ^{33}P uptake in the polysaccharide and residual fractions was linear, ANCOVAs were performed to determine differences among treatments. However, there were no differences in ^{33}P incorporation between the PI and PB treatments in the polysaccharide fraction of *A. tonsa* (df=77, F=0.69, p=0.409), but the residual fraction demonstrated a difference between treatments (df=78, F=8.56, p=0.005). A one-way ANOVA was performed on the data from the saturated portion (173+ hours) of the RNA fraction during uptake, which resulted in no difference between treatments (df=71, F=0.15, p=0.705). No statistical comparison was performed for the protein fraction as it

saturated in one case and remained linear in the other. Thus, they were not amenable for statistical comparison (i.e., neither the linearity assumption of the ANCOVA applied, nor did the signal stabilize as required for an ANOVA).

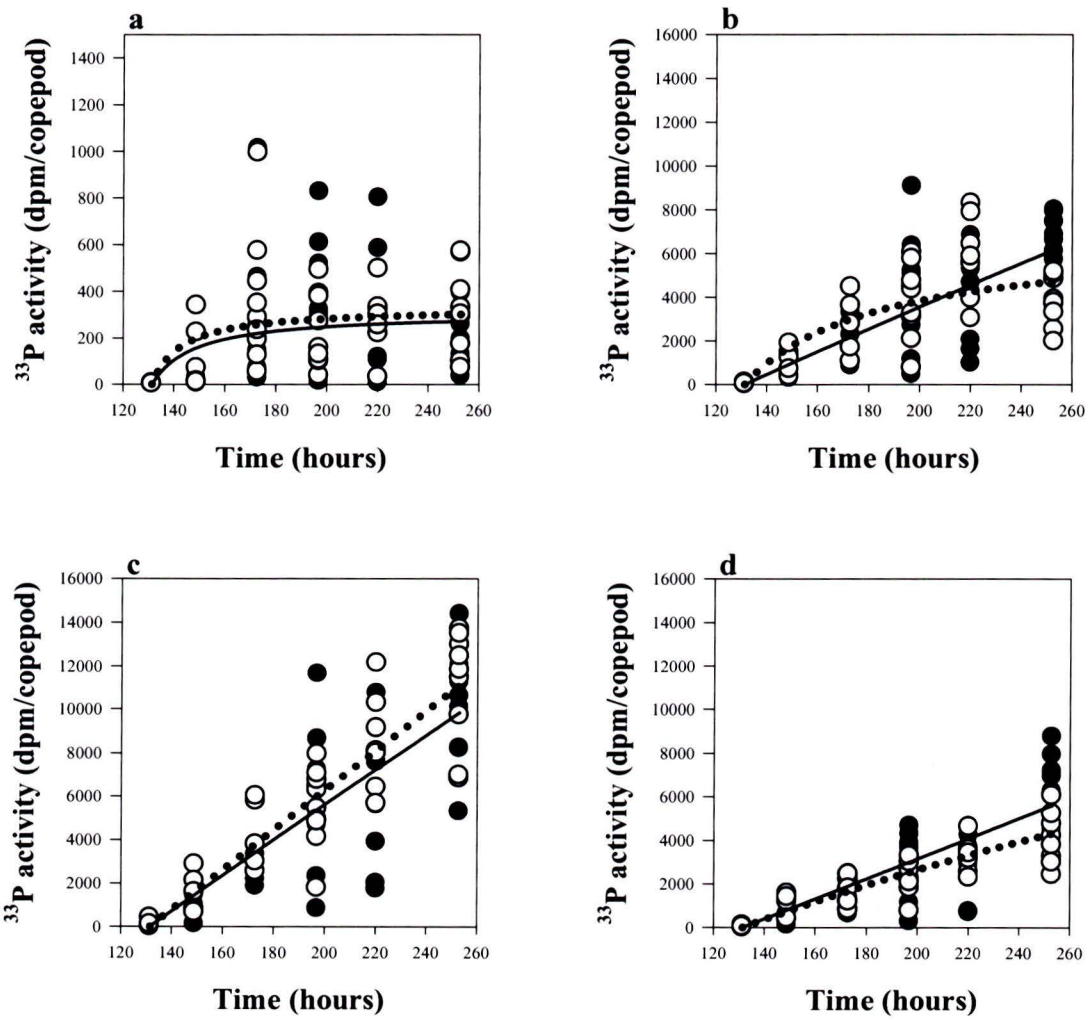

Fig. 22. ^{33}P activity in biochemical fractions of *A. tonsa* during the ^{33}P incorporation experiment time-series. Panels are RNA (a), protein (b), polysaccharide (c) and residuals (d) with filled circles indicating P-imbalanced (PI) and open circles indicating P-balanced (PB) fed *A. tonsa*. Saturation or incorporation lines are solid for PI fed and dotted for PB fed *A tonsa*. Note that the scale for RNA is different from the other fractions due to its comparatively low values. Copepod samples were taken as individuals with 7-10 samples taken per treatment at every time-point.

Particulate phosphorus and Chlorophyll a content of Rhodomonas salina and Thalassiosira weissflogii during the ^{33}P elimination experiment

Pre-labeled populations of P-imbalanced (PI) and P-balanced (PB) *Acartia tonsa* (see Methods) were placed in three food environments (ASW-P (no food)/*Rhodomonas salina*/*Thalassiosira weissflogii*) to determine ^{33}P elimination from the copepod under different feeding regimes. Particulate phosphorus and Chl *a* values from the *R. salina* and *T. weissflogii* food environments are presented on a volumetric basis (Fig. 23), due to problems with cell counts in *T. weissflogii*. ASW-P values are not presented here, as those environments did not contain food. One-way ANOVAs were performed on particulate phosphorus and Chl *a* with no significant differences found between treatments (pre-fed PI or PB *R. salina*) within the *T. weissflogii* feeding chambers (Table 11). In the *R. salina* feeding chambers, however, between treatment differences occurred in Chl *a* (Table 11). Yet it is apparent that *T. weissflogii*'s particulate phosphorus and Chl *a* values were greater than *R. salina*'s and ultimately resulted in significant differences in both particulate phosphorus and Chl *a* values in most instances (Fig. 23, Table 12). The *T. weissflogii* food environments contained about 3.7-3.9 times more particulate phosphorus and 4.4-4.9 times more Chl *a* than the *R. salina* food environments (Table 12).

^{33}P elimination from Acartia tonsa

All pre-fed treatments (PI or PB) and food environments (ASW-P (no food)/*Rhodomonas salina*/*Thalassiosira weissflogii*) demonstrated a decrease in radioactivity (^{33}P dpm) (Table 13) during the elimination experiment as an overall loss of ^{33}P from *Acartia tonsa* tissues (Figs. 24-25). For comparison, and following the

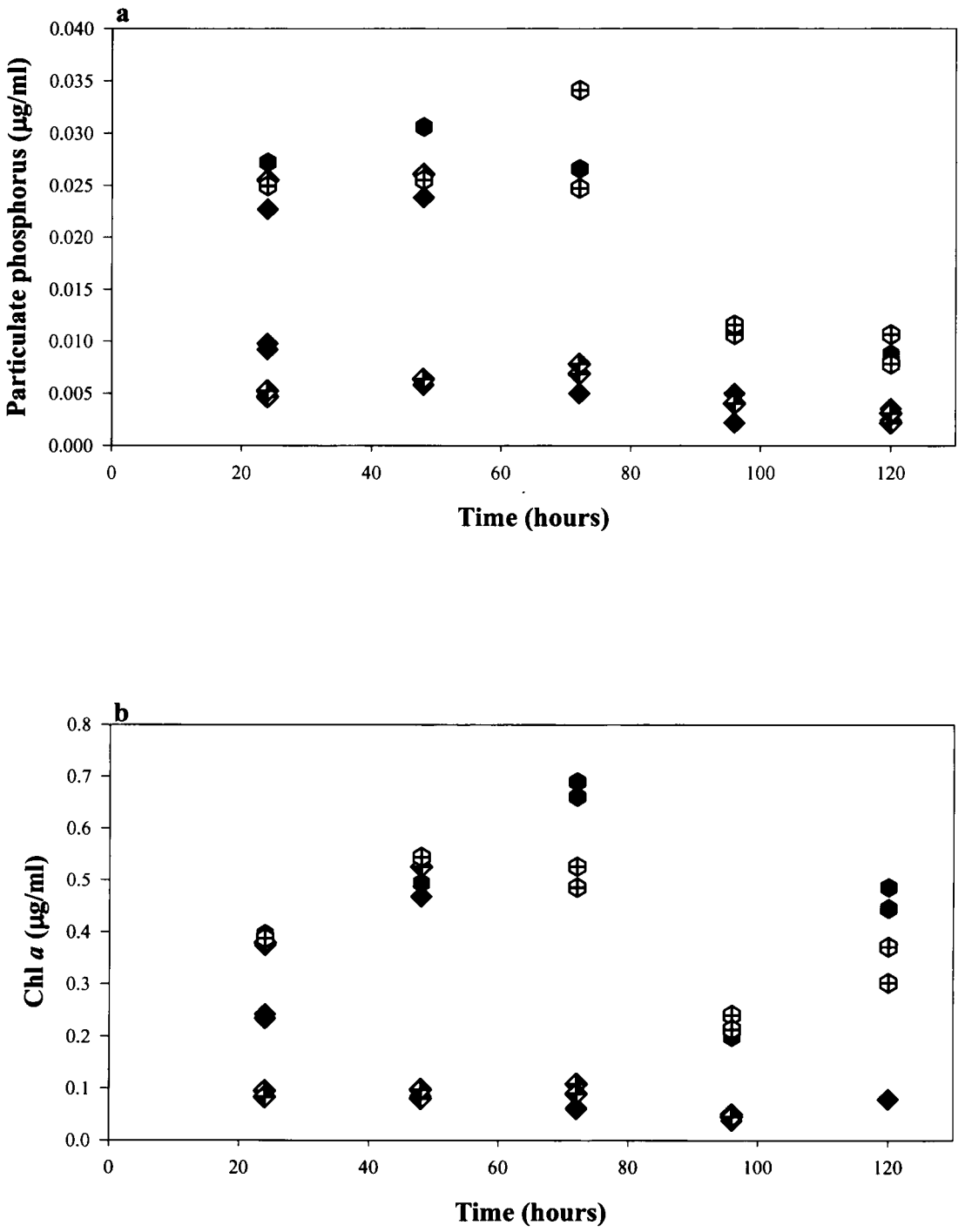

Fig. 23. Volume specific values of particulate phosphorus (a) and Chl *a* (b) during the ^{33}P elimination experiment time-series for non-radioactive *R. salina* and *T. weissflogii* presented to the pre-labeled *A. tonsa*. Filled symbols indicate algae presented to P-imbalanced (PI) pre-fed copepods and open symbols indicate those algae presented to P-balanced (PB) pre-fed copepods. *R. salina* is represented by diamonds and *T. weissflogii* by hexagons. Sample volumes were 15-25 ml for particulate phosphorus and 4-5 ml for Chl *a*.

Table 11
Means and standard deviations for the volumetric values of particulate phosphorus and Chl *a* for *R. salina* and *T. weissflogii* food environments during the ^{33}P elimination experiment time-series. PI and PB indicate the feeding histories of *A. tonsa* (pre-fed P-imbalanced (PI) and P-balanced (PB) *R.salina*). Results of pair-wise (between treatment), one-way ANOVAs performed on each measurement are given in the last column. Those ANOVAs with p-values <0.05 are in bold.

Food environment	Chemical constituents	PI treatment			PB treatment			ANOVA	
		n	Mean	Standard deviation	n	Mean	Standard deviation	F	p-value
R. salina									
	Particulate phosphorus (µg/ml)	10	0.005	0.0025	10	0.005	0.0018	4.30	0.055
	Chl *a* (µg/ml)	9	0.11	0.078	8	0.08	0.022	6.49	**0.024**
T. weissflogii									
	Particulate phosphorus (µg/ml)	10	0.020	0.0087	10	0.020	0.0091	<0.01	0.990
	Chl *a* (µg/ml)	10	0.44	0.160	10	0.40	0.120	0.07	0.790

Table 12
Means and standard deviations for the volumetric values of particulate phosphorus and Chl *a* for *R. salina* and *T. weissflogii* food environments during the ^{33}P elimination experiment time-series. PI and PB indicate the feeding histories of *A. tonsa* (pre-fed P-imbalanced (PI) and P-balanced (PB) *R.salina*). Results of pair-wise (between algae), one-way ANOVAs performed on each measurement are given in the last column. Those ANOVAs with p-values <0.05 are in bold.

Pre-fed	Chemical constituent	*R. salina*			*T. weissflogii*			ANOVA	
		n	Mean	Standard deviation	n	Mean	Standard deviation	F	p-value
PI									
	Particulate phosphorus (µg/ml)	10	0.005	0.0025	10	0.020	0.0087	38.00	**<0.001**
	Chl *a* (µg/ml)	9	0.11	0.078	10	0.44	0.160	3.23	0.093
PB									
	Particulate phosphorus (µg/ml)	10	0.005	0.0018	10	0.020	0.0091	33.07	**<0.001**
	Chl *a* (µg/ml)	8	0.08	0.022	10	0.40	0.120	17.79	**0.001**

exponential decay model shown in Fig. 1b, the radioactivity data was natural-log transformed and plotted against time. At t_0, in both treatments, poly-na had the highest ^{33}P values followed by LMW compounds, protein and lipid fractions in decreasing order (Figs. 24-25). Pair-wise ANCOVAs were then performed to test for the homogeneity of slopes among various treatments. Feeding history, being pre-fed PI or PB *R. salina*, had no effect on the slopes of ^{33}P elimination from *A. tonsa* in all biochemical fractions and the totals (sum of all fractions) (Tables 13-14), except for the lipid fraction in the ASW-P treatment ($df=23$, $F=5.27$, $p=0.03$). No correction was applied for multiple pair-wise comparisons (see Nakagawa 2004 for a discussion). However, the most extreme Bonferroni correction for the criterion α (i.e., $\alpha_{corr} = \alpha/k=0.05/12=0.0042$, where k is the number of pair-wise comparisons) would raise the level of significance below the p-value of the lipid test. The significant effect of the lipid fraction could thus have arisen by chance leaving the result for this particular biochemical fraction inconclusive.

Table 13
^{33}P elimination rates (slopes) of protein, LMW compounds, lipid and poly-na fractions from P-imbalanced (PI) and P-balanced (PB) *R. salina* pre-fed *A. tonsa* from the ASW-P (i.e., no food), *R. salina* and *T. weissflogii* food environments. Elimination rate represents the slopes of linear regression of the natural log-transformed data shown in Figs. 24 and 25.

Biochemical fraction	**ASW-P (ln dpm/copepod/hr)**		***R. salina*** **(ln dpm/copepod/hr)**		***T. weissflogii*** **(ln dpm/copepod/hr)**	
Pre-fed treatment	**PI**	**PB**	**PI**	**PB**	**PI**	**PB**
Protein	-0.0053	-0.0065	-0.0084	-0.0075	-0.012	-0.013
LMW compounds	-0.0044	-0.0044	-0.0064	-0.0062	-0.011	-0.010
Lipid	-0.0028	-0.0058	-0.0061	-0.0064	-0.011	-0.011
Poly-na	-0.0075	-0.0067	-0.0088	-0.0070	-0.012	-0.013
Totals	-0.0052	-0.0057	-0.0074	-0.0068	-0.012	-0.012

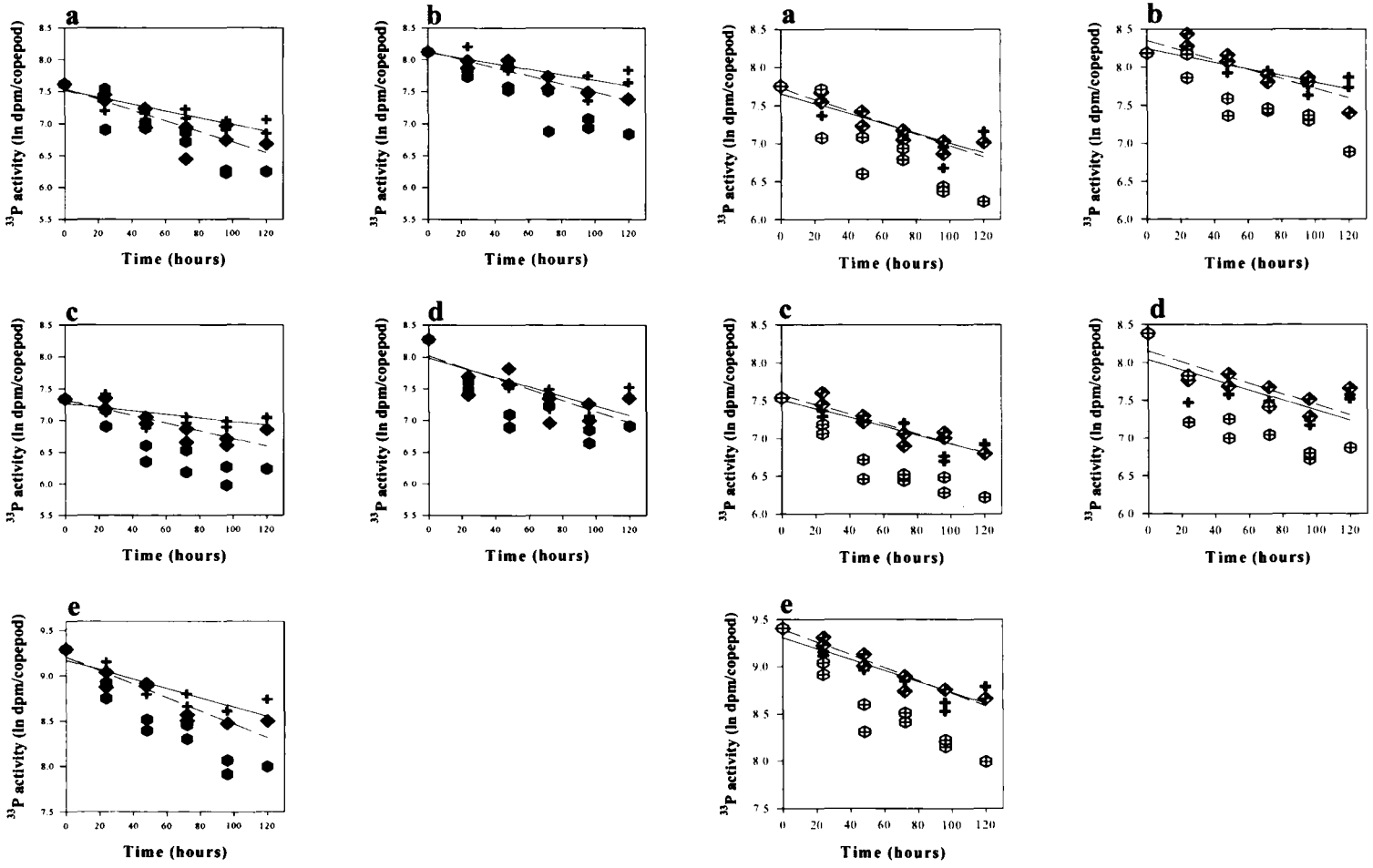

Fig. 24 (L) and Fig. 25 (R). ^{33}P activity in biochemical fractions of P-imbalanced (PI) (L) and P-balanced (PB) (R) pre-fed *A. tonsa* and totals (sum of all fractions) during the ^{33}P elimination experiment time-series. Panels are (left to right) protein (a), LMW compounds (b), lipid (c), poly-na (d) and totals (e). Filled symbols indicate PI fed *A. tonsa* and open symbols indicate PB fed *A. tonsa*. Symbols are plusses (ASW-P, i.e., no food), diamonds (non-radioactive *R. salina*) and hexagons (non-radioactive *T. weissflogii*). Note natural log scale on Y-axis. Slopes of elimination (solid (ASW-P), dashed (non-radioactive *R. salina*) and dotted (non-radioactive *T. weissflogii*) were variable between fractions and in the totals of the various food environments (ASW/*R. salina*/*T. weissflogii*), according to a test of homogeneity of slopes using the ANCOVA procedure (Table 13). Copepod samples consisted of 1-7 individuals per filter.

Table 14
Summary results of pair-wise ANCOVAs for the effect of feeding history (ingesting P-imbalanced (PI) or P-balanced (PB) *R. salina*) on ^{33}P elimination rates from *A. tonsa* in different food environments (ASW-P/*R. salina*/*T. weissflogii*). Those ANCOVAs with p-values <0.05 are in bold.

Biochemical fraction	ASW-P			*R. salina*			*T. weissflogii*		
	df	F	p-value	df	F	p-values	df	F	p-value
Protein	23	0.63	0.44	21	0.20	0.66	21	0.06	0.82
LMW compounds	23	<0.01	0.99	21	0.01	0.92	21	0.47	0.50
Lipid	23	5.27	**0.03**	21	0.06	0.82	21	0.02	0.90
Poly-na	23	0.09	0.77	21	0.38	0.54	21	0.08	0.78
Totals	23	0.17	0.69	21	0.37	0.55	21	<0.01	0.96

In an attempt to further understand phosphorus turnover mechanisms in *A. tonsa*, fractional comparisons within each pre-fed treatment and food environment were analyzed. Pair-wise ANCOVAs performed on the ^{33}P elimination data revealed no significant differences among fractional sets (Table 15). Contrary to the variable phosphorus turnover rates in the ^{33}P incorporation, all fractions eliminated ^{33}P at similar rates within each pre-fed treatment and food environment.

Table 15
Summary results of pair-wise ANCOVAs for comparing ^{33}P elimination rates between biochemical fractions within P-imbalanced (PI) and P-balanced (PB) pre-fed *A. tonsa* from different food environments (ASW-P/*R. salina*/*T. weissflogii*). Those ANCOVAs with p-values <0.05 are in bold.

Pre-fed treatment	Biochemical fraction	ASW-P			*R. salina*			*T. weissflogii*		
		df	F	p-value	df	F	p-value	df	F	p-value
PI										
	Protein vs. LMW compounds	23	0.40	0.53	21	1.26	0.28	21	0.33	0.58
	Protein vs. Lipid	23	3.26	0.09	21	1.34	0.26	21	0.37	0.55
	Protein vs. Poly-na	23	1.01	0.33	21	0.02	0.89	21	0.05	0.82
	LMW compounds vs. Lipid	23	1.09	0.31	21	0.05	0.82	21	0.01	0.91
	LMW compounds vs. Poly-na	23	1.89	0.18	21	1.05	0.32	21	0.03	0.86
	Lipid vs. Poly-na	23	4.35	0.05	21	1.16	0.30	21	0.06	0.81

Table 15 Continued

Pre-fed treatment	Biochemical fraction	ASW-P			*R. salina*			*T. weissflogii*		
PB		**df**	**F**	**p-value**	**df**	**F**	**p-value**	**df**	**F**	**p-value**
	Protein vs. LMW compounds	23	2.19	0.15	21	0.81	0.38	21	1.74	0.20
	Protein vs. Lipid	23	0.21	0.65	21	0.85	0.37	21	0.49	0.49
	Protein vs. Poly-na	23	0.01	0.93	21	0.07	0.79	21	<0.01	0.95
	LMW compounds vs. Lipid	23	1.52	0.23	21	0.02	0.90	21	0.44	0.52
	LMW compounds vs. Poly-na	23	1.28	0.27	21	0.16	0.70	21	0.88	0.36
	Lipid vs. Poly-na	23	0.18	0.68	21	0.11	0.74	21	0.23	0.64

Each food category produced identical ^{33}P elimination patterns for both the PI and PB pre-fed copepods (Figs. 24-25), rendering the two treatments replicates for the purpose of comparing food treatments. Thus, no difference in elimination rates among biochemical fractions or among pre-feeding treatments occurred. However, there were significant differences in the elimination rates depending on the three food treatments (i.e., ASW-P/*R. salina*/*T. weissflogii*). The effect of food environments on ^{33}P elimination rates was determined by additional pair-wise ANCOVAs performed by fraction in each pre-fed treatment. The strongest difference in elimination patterns was observed between *T. weissflogii* and ASW-P, and to a lesser extent between the *T. weissflogii* and *R. salina* food environments (Figs. 24-25; Table 16). Elimination slopes were significantly different only between the *R. salina* and ASW-P food environments in the lipid fraction of the PI pre-fed treatment (Table 16). Clear delineations in ^{33}P elimination rates between ASW-P, *R. salina* and *T. weissflogii* food environments were seen in the PI pre-fed copepods in the protein and lipid fractions (Figs. 24a and 24c), as well as in the totals (Fig. 24e). However, in the LMW compounds and poly-na fractions,

the differences were less apparent, but more evident was the separation of *T. weissflogii* from the other two environments (Figs. 24b and 24d). The apparent differences between *T. weissflogii* and the other two environments (ASW-P and *R. salina*) were supported by the resulting ANCOVAs (Table 16). With the exception of the lipid fraction, there were no significant differences in the ASW-P vs. *R. salina* comparison in any other fraction or in the totals (Table 16). Also, significant differences occurred in every comparison, including the totals, except the poly-na fraction of ASW-P vs. *T. weissflogii* comparison. Yet for the *R. salina* and *T. weissflogii* comparison, only the LMW compounds, lipid and totals were found to be significantly different (Table 16).

Table 16
Summary results of pair-wise ANCOVAs for comparing ^{33}P elimination rates between food environments (ASW-P/*R. salina*/*T. weissflogii*) for each biochemical fraction within each treatment (pre-feeding P-imbalanced (PI) and P-balanced (PB) *R. salina*). Those ANCOVAs with p-values <0.05 are in bold.

Pre-fed treatment	Biochemical fraction	ASW-P vs. *R. salina*			ASW-P vs. *T. weissflogii*			*R. salina* vs. *T. weissflogii*		
		df	F	p-value	df	F	p-values	df	F	p-value
PI										
	Protein	22	2.85	0.1100	22	16.81	**0.0006**	21	3.12	0.0940
	LMW compounds	22	2.30	0.1500	22	16.30	**0.0007**	21	10.70	**0.0042**
	Lipid	22	5.04	**0.0370**	22	18.29	**0.0004**	21	5.62	**0.0290**
	Poly-na	22	0.17	0.6900	22	1.71	0.2100	21	0.76	0.3900
	Totals	22	1.14	0.3000	22	12.50	**0.0020**	21	7.78	**0.0120**
PB										
	Protein	22	0.46	0.5000	22	8.82	**0.0079**	21	6.84	**0.0180**
	LMW compounds	22	0.19	0.1900	22	15.76	**0.0008**	21	4.83	**0.0410**
	Lipid	22	0.24	0.6300	22	10.61	**0.0041**	21	8.020	**0.0110**
	Poly-na	22	0.02	0.8900	22	3.51	0.0760	21	3.26	0.0880
	Totals	22	0.07	0.8000	22	10.37	**0.0050**	21	11.43	**0.0030**

Unlike the case for the PI pre-fed copepods, the elimination of ^{33}P from the PB pre-fed copepods did not result in a clear separation of all three food environments (Fig. 25). The ^{33}P elimination rates in the ASW-P and *R. salina* food environment appear nearly identical and much slower than in the *T. weissflogii* food environment (Fig. 25; Table 13); this was supported by the ANCOVA results for ASW-P vs. *R. salina* comparison, as there were no significant differences in any fraction or in the totals (Table 16). Yet again, the main differentiation between food environments came when *T. weissflogii* was being compared to any other environment (Fig. 25) with protein, LMW compounds, lipid and totals being found significant in each comparison (Table 16). Further support for *T. weissflogii* inducing greater elimination of ^{33}P was seen in the time taken by each fraction to expel 50% of its radioactivity (Table 17). In averaging the totals, *T. weissflogii* had the lowest time at 58 hours, which was followed by *R. salina* at 91 hours and ASW-P at 104 hours. ^{33}P elimination rates were thus almost twice as fast when the copepods fed on *T. weissflogii* than when copepods were kept without food, while *R. salina* was equidistant from the other two treatments. Overall, the elimination of ^{33}P from the poly-na fraction was 1.3-2.7 times faster than in other fractions of any food environment (Table 17).

Table 17
Calculated time required (in hours) for P-imbalanced (PI) and P-balanced (PB) pre-fed *A. tonsa* to eliminate half of their maximum ^{33}P signal (dpm) based on food environment (ASW-P/*R. salina*/*T.weissflogii*).

	ASW-P		***R salina***		***T. weissflogii***	
Biochemical fraction	**PI (hrs)**	**PB (hrs)**	**PI (hrs)**	**PB (hrs)**	**PI (hrs)**	**PB (hrs)**
Protein	108.1	94.0	79.4	89.6	63.1	59.5
LMW compounds	136.6	141.4	106.8	109.5	66.7	74.1
Lipid	173.8	111.8	106.2	105.1	62.3	57.5
Poly-na	64.6	63.2	60.2	71.2	46.6	42.7
Totals	106.7	101.0	83.6	98.5	58.5	57.5

Additionally, two other distinct patterns from the experiment were apparent. The ^{33}P elimination rate of the poly-na fraction, of all food environment comparison sets, was never found to be significantly different (Table 16). Conversely, the ^{33}P elimination rate in the lipid fraction was significantly different in almost all food environment comparisons (Table 16). This means that overall the lipid fraction was most sensitive, and the poly-na fraction of *A. tonsa* was least sensitive to the biochemical changes in the food.

The percentage of ^{33}P distributed among biochemical fractions from all food environments (ASW-P/*R. salina*/*T.weissflogii*) are displayed in Fig. 26. The comparisons between treatment percentages for each fraction resulted in no significant differences (Table 18). Moreover, the percentages were very consistent between pre-fed treatments (PI/PB), among food environments (ASW-P/*R. salina*/*T.weissflogii*), and with time (Fig. 26) with average values of 18-19% ^{33}P - protein, 34-37% ^{33}P - LMW compounds, 14-17% ^{33}P - lipid and 26-30% ^{33}P - poly-na. Incorporation and elimination of ^{33}P occurred proportionally and independently of the biochemical fraction.

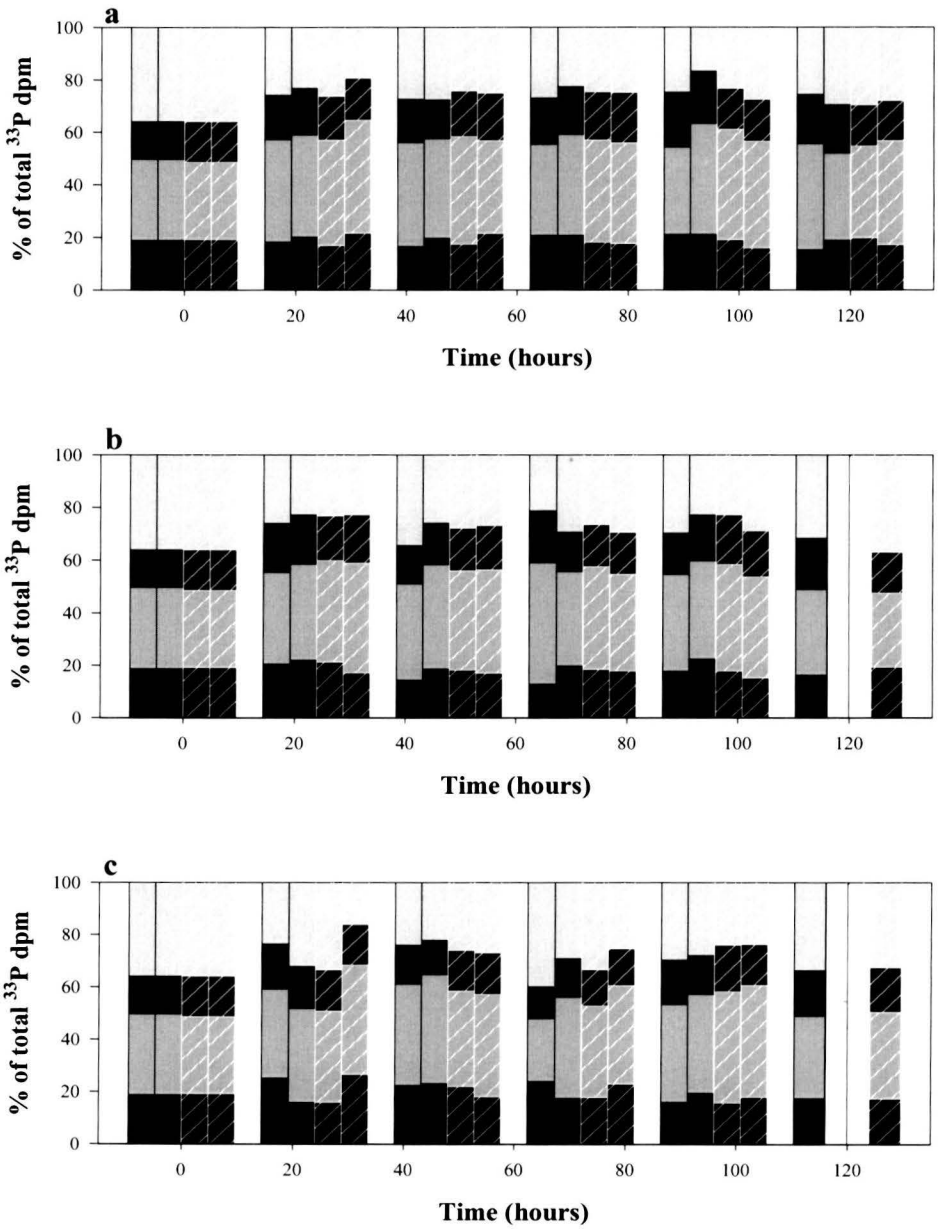

Fig. 26. Percentages of ^{33}P in the biochemical fractions of P-imbalanced (PI) and P-balanced (PB) pre-fed *A. tonsa* during the ^{33}P elimination experiment time-series. Panels are ASW-P (i.e., no food) (a), non-radioactive *R. salina* (b) and non-radioactive *T. weissflogii* (c). Solid bars represent P-imbalanced (PI), while hatched lines represent P-balanced (PB) *A. tonsa*. The fractions are (from bottom to top): protein, LMW compounds, lipid and poly-na.

Table 18

Means and standard deviations for percent ^{33}P remaining in P-imbalanced (PI) and P-balanced (PB) pre-fed *A. tonsa* tissues after being placed into ASW-P, *R. salina* and *T. weissflogii*. Results of pair-wise (between treatment), one-way ANOVAs for the effect of feeding history (pre-fed PI or PB *R. salina*) are given in the last column. No fraction was significantly different as all p-values were >0.05.

		PI			PB			ANOVA	
Food Environment	**Biochemical fraction**	**n**	**Mean**	**Standard deviation**	**n**	**Mean**	**Standard deviation**	**F**	**p-value**
ASW-P									
	Protein	12	19.06	1.84	12	18.51	1.78	0.55	0.47
	LMW compounds	12	36.71	3.83	12	37.76	4.55	0.37	0.55
	Lipid	12	17.25	2.14	12	16.64	1.35	0.70	0.41
	Poly-na	12	26.99	5.36	12	27.10	4.89	<0.01	0.96
R. salina									
	Protein	11	18.30	2.96	11	18.19	1.55	0.01	0.91
	LMW compounds	11	36.38	4.32	11	36.41	4.90	<0.01	0.99
	Lipid	11	16.49	2.12	11	16.57	1.13	0.02	0.91
	Poly-na	11	28.83	5.35	11	28.83	5.29	<0.01	1.00
T. weissflogii									
	Protein	11	19.80	3.24	11	19.32	3.23	0.12	0.73
	LMW compounds	11	34.77	5.01	11	36.55	4.76	0.73	0.40
	Lipid	11	14.90	1.66	11	15.53	1.12	1.10	0.31
	Poly-na	11	30.54	5.73	11	28.60	6.26	0.57	0.46

DISCUSSION

Dissolved Phosphorus

Dissolved phosphorus concentrations from the algae incorporation experiments were similar across experiments. Both incorporations began with elevated concentrations (~2.5 μM – Fig. 11a; ~2.4-2.7 μM – Fig. 19a) and immediately plummeted to low concentrations (~0-0.04 μM), the result of a significant phosphorus uptake (Figs. 11b and 19b). Cellular phosphorus uptake for *Rhodomonas salina* averaged between 0.49-1.03 fmol/cell/hr in the PI algae and 0.63-0.79 fmol/cell/hr in the PB algae. Low dissolved phosphorus values, like those presented here, were found in annual average phosphorus concentrations in the surface waters of world's oceans in 2005 between 45°N - 45°S (0.05-0.50 μM: NOAA 2005), which agrees with some authors' theory that marine waters are deplete in P or are P-limited (Karl et al., 2001; Downing, 1997) (See Introduction).

Particulate phosphorus and chlorophyll a values for Rhodomonas salina

The present work represents an improvement over many previous experiments in that the phosphorus imbalance was not created by the lack of phosphorus in one treatment versus the other, but instead phosphorus levels were kept exactly equal between treatments, and all remaining nutrients were altered instead. This method circumvented the problem of the effect of bulk phosphorus on isotopic dilution and growth characteristics. The excess of other nutrients in one treatment (PI), in effect, created a phosphorus limited media by comparison. This concept is supported by Lenton and Klausmeier (2006), who stated that phytoplankton with a N:P ratio of 45 or more are

considered phosphorus limited. Considering PI *Rhodomonas salina* had a N:P of 61, it should be regarded as phosphorus limited.

With two distinct media used for culturing, P-imbalanced (PI) with phosphorus at f/20-Si concentrations and remaining nutrients at f/2-Si and P-balanced (PB) with all nutrients at f/20-Si concentration (Appendix IV), the resulting PI and PB *R. salina* displayed both similarities and differences in chemical composition. Cellular Chl *a* values were visibly and statistically different between the two treatments in both incorporation experiments (Figs. 12c, 13 and 20b; Tables 2 and 9) with PI cultured *R. salina* having greater Chl *a* content. Average cell specific Chl *a* values reflected a similar difference of 3.5 to 4.6 times greater values in the PI cultured *R. salina* (Figs. 12c and 20b; Tables 2 and 9). Average Chl *a* values (~1 pg Chl *a*/cell) for PI cultured *R. salina*, in this study, were similar to those found by Coutteau (1996) for *Chroomonas salina* (a previous name for *Rhodomonas salina*), at 1.0 pg Chl *a*/cell. Similarly, Eriksen et al. (1998) stated that *Rhodomonas* sp. with values of 1.5 pg Chl *a* /cell were nutrient sufficient. Lafarga-De la Cruz et al. (2006) found values of 1.35 ±0.015 pg Chl *a*/cell in *Rhodomonas* spp. Lower values, like those found in Lewitus and Caron (1990) with phosphorus limited *Pyrenmonas salina* (another previous name for *Rhodomonas salina*), produced cells with 0.42 pg Chl *a*/cell at early stationary phase, but up to 0.75 pg Chl *a*/cell at early exponential. Thus, the higher cell specific Chl *a* values, in the above studies, from generally high nutrient media, were more akin to the PI cultured algae, while the lower values from nutrient limiting situations were similar to the PB cultured algae. The molecular formula of Chl *a* is $C_{55}H_{72}MgN_4O_5$ (Gossauer and Engel, 1996), and the PI medium, being deficient only in phosphorus by comparison to other nutrients,

provided sufficient nutrients to build large amounts of Chl *a* per cell. It follows that the PI cultured *R. salina* were able to build many substances, other than Chl *a*, that are rich in carbon and nitrogen (Figs. 20a and 20c), and that do not require phosphorus, resulting in higher C:P and N:P ratios (Figs. 21b-c).

Cellular particulate phosphorus values were not as clearly divided, with *R. salina* (I) being significantly different and *R. salina* (II) was not (Figs. 12b and 20e; Tables 2 and 9). However, it is likely that the average for PB *R. salina* (I) was driven up by the value at 115 hours (Fig. 12b) and that the two treatments were not really different. It was expected that both algae would contain the same amount of phosphorus because equal amounts were available to the algae in both treatments.

General pools of phosphorus in algae and copepods

Before discussing the implications of the results from biochemical fractionations, it is important that sources of phosphorus, whether obvious or obscure, be presented. Figure 27a shows the N and P percentages of various cell components; of particular interest for this study were chloroplast (~0.25% elemental P), mitochondria (~0.25% elemental P), endoplasmic reticulum (~1.15% elemental P), animal plasmalemma (~1.25% elemental P) and eukaryotic ribosomes (~5% elemental P). Sterner and Elser (2002) also illustrated the differing percentages of N and P in important compounds such as phospholipids, ATP, and nucleic acids, which contain 4%, 18% and 8.7% phosphorus (elemental %P), respectively (Fig. 27b). Note that proteins, chlorophyll and chitin – essential substances in the present study – according to Sterner and Elser (2002), contain no phosphorus (Fig. 27b). However, the existence of compounds like phosphoproteins and hydroxyapatite (a mineral associated with the copepod carapace), can add

phosphorus to these compound groups, and would change the idealized representation in Fig. 27b.

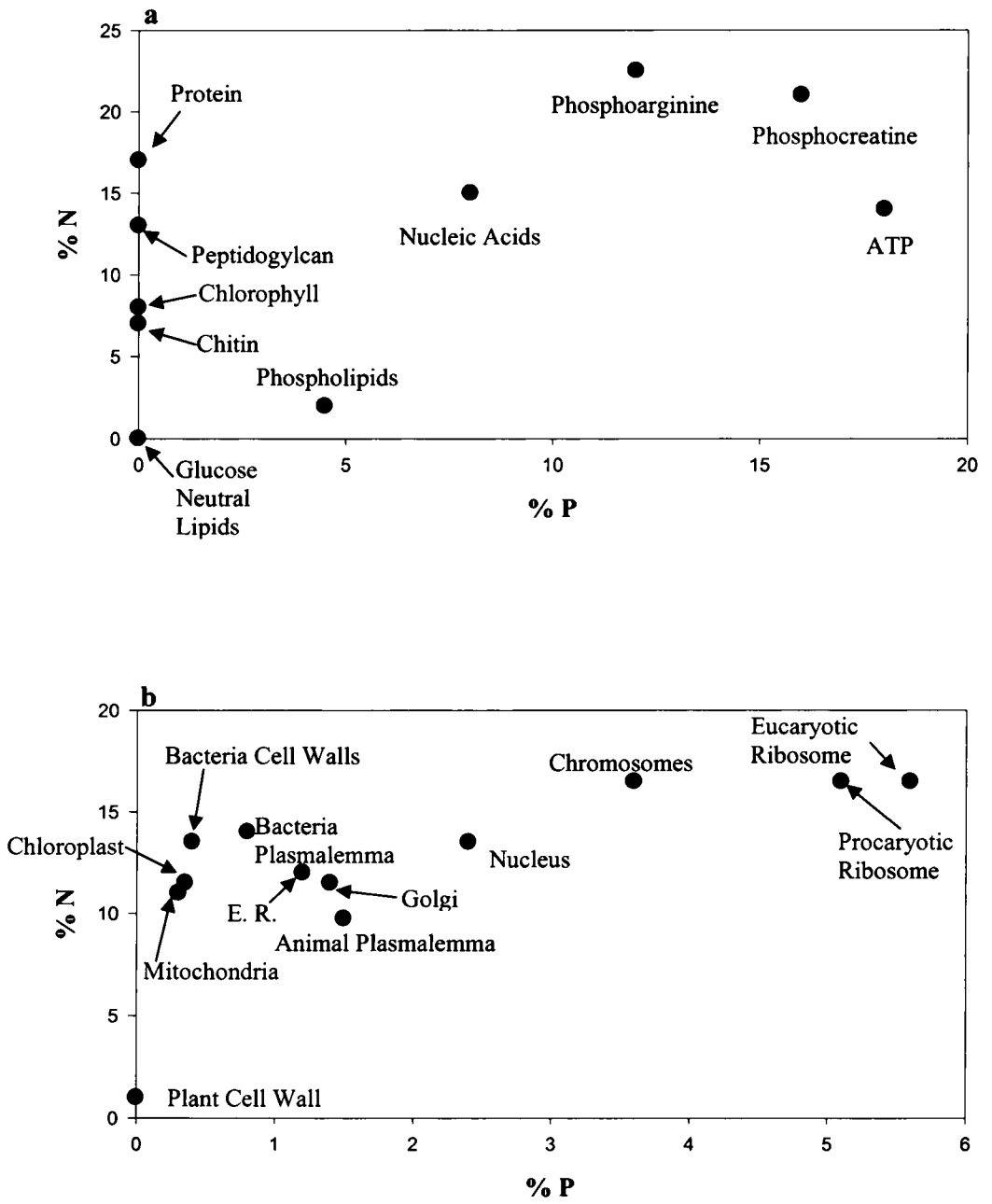

Fig. 27. Redrawn from Sterner and Elser (2002), percent phosphorus and nitrogen of (a) various cellular components of bacteria, plants and animals and (b) various compounds found in living organisms.

Relative distribution of ^{33}P in biochemical fractions of Rhodomonas salina

After 42 hours, the P-balanced (PB) cultured *Rhodomonas salina* had greater, relative to P-imbalanced (PI) cultured algae, average radioactivity in all fractions and the totals except for the low molecular weight fraction (Fig. 14; Table 3). The amount of ^{33}P incorporated into the biochemical fractions ranked, based on Fig. 14a-d, (in descending order): protein, polysaccharide-nucleic acid, LMW compounds and lipid for both PI and PB cultured algae.

The average ^{33}P activity in the protein fraction of *R. salina* was greater than would have been predicted because proteins do not typically contain phosphorus (contrast Figs. 14a and 27b). The existence of phosphoproteins within Chl *a* found in the algal cell could account for the higher values observed here. Bennett (1979) found that pea chloroplasts incorporate ^{32}P into their proteins. This phosphoprotein was determined to be a peripheral protein in the thylakoid membrane, which in the presence of light became phosphorylated (Bennett, 1979). During the phosphorylation, the phosphoprotein is partially released from the membrane (Carlberg et al., 2003; Hansson et al., 2007). Since the experiments in this thesis were performed under continuous light, it could have accounted for the high ^{33}P values in the protein fraction. An additional, more sobering, possibility was that the *R. salina* cells were never broken up sufficiently and ^{33}P from other fractions did not dissolve completely, remaining on the filter even after hot TCA treatment. This scenario was not likely to be the case as those studies on which the current protocol was based did not require maceration of algal cells (Bochdansky et al., 1999; Roman, 1991; Rivkin, 1985; Li et al., 1980). Furthermore, this would be particularly troubling as the method used was exactly the same as the one employed for

approximately 40 years (Li et al., 1980). A main difference in methods of Bochdansky et al. (1999), Roman (1991) and Rivkin (1985) and the present study was the use of ^{33}P instead of ^{14}C, a fact that makes high values in the protein fraction more conspicuous. Of the total amount of ^{33}P or ^{14}C incorporated, ~50 % of each isotope was incorporated into the protein fraction (Fig. 15; Table 19). Regardless of the mechanism that allowed for the appearance of substantial ^{33}P radioactivity in the protein fraction, the PB cultured *R. salina* contained, on average, ~2 times more ^{33}P than the PI cultured algae (Table 3).

Table 19
Percent ^{14}C incorporation into protein, LMW compounds, lipid and poly-na fractions of various plankton based on literature values. The fraction with the greatest percentage of ^{14}C within each organism is in bold.

Organism	Protein	LMW compounds	Lipid	Poly-na	Source
Laboratory cultured *Skeletonema costatum*	33	22	**35**	10	Rivkin (1985)
Thalassiosira rotula	**48**	15	31	6	"
Laboratory cultured *Ditylum brightwelli*	**41**	30	22	7	"
Pyrocystis noctiluca	31	26	**33**	10	"
Gonyaulax polyedra	**47**	16	24	13	"
Prorocentrum lebouriae	**34**	21	32	13	"
Phytoplankton coastal assemblage	**40**	18	28	13	"
Ceratium tripos	**49**	13	20	18	"
Field cultured *Ditylum brightwelli*	**54**	19	15	10	"
Phytoplankton estuarine assemblage	**40**	20	28	11	"
Heterocapsa triquerta	**44**	16	20	19	"
Field cultured *Skeletonema costatum*	**52**	21	18	9	"
Thalassiosira nordenskioldii	**54**	17	18	11	Bochdansky et al. (1999)
Oikopleura vanhoeffeni	10	**67**	18	5	"
Blastocladiella emersonii	19	<9	7	**60.4**	Cantino (1965)
Oscillatoria thiebautii	15-18	22-25	2-3	**50-60**	Li et al. (1980)

Table 19 Continued

Organism	Protein	LMW compounds	Lipid	Poly-na	Source
Phaeodactylum tricornutum	17-25	25-40	-	**30-55**	Morris et al. (1974)
Acartia tonsa	20-22	<20	18-21	**50-58**	Putland (2005)
Laboea strobila	5	18	19	**58**	Putt (1990)
Thalassiosira weissflogii	**56**	14.4	21.2	8.6	Roman (1991)
Adult *Acartia tonsa*	35	**40**	15	15	"
Southern Ocean phytoplankton at 10°C	10	-	30	**60**	Smith and Morris (1980)
Scenedesmus (P-)	**54.1**	6.6	9.7	29.6	DeMott et al. (1998)
Tetraselmis impellucida	**39**	15	**39**	8	Thor et al. (2002)
Dunaliella tertiolecta	**39**	15	**39**	6	"
Acartia tonsa fed *T. impellucida*	**48**	38	3	13	"
Acartia tonsa fed *D. tertiolecta*	26	**33**	**33**	12	"
Natural phytoplankton assemblage in Apalachicola Bay	20-25	<20	20	**50-57**	Putland and Iverson (2007)
Scenedesmus (P+)	**65.3**	6.0	10.3	18.5	"
Calanus finmarchicus	9	-	17.8	**73.7**	Corner (1973)

The poly-na fraction was expected to contain a significant amount of phosphorus and indeed, it contained the second highest amount of ^{33}P. PB cultured algae contained 1.7 times more ^{33}P than the PI cultured algae (Table 3) The poly-na fraction in these experiments not only contained polysaccharides, which are typically P poor, consisting of compounds like cellulose $(C_6H_{10}O_5)_n$ and chitin $(C_8H_{13}O_5N)_n$ (Allinger et al., 1971), but also nucleic acids, which are P rich as they typically are 8.7% P (molecular %P) (Fig. 27b). Furthermore, Sterner and Elser (2002) stated that small organisms have a large proportion of phosphorus in nucleic acids, especially RNA. In the nucleic acid portion, DNA and mRNA codons add minute quantities of phosphorus with 6 phosphates per

amino acid of the DNA codon and 3 phosphates per amino acid of mRNA (Sterner and Elser, 2002). tRNA and rRNA, on the other hand, contribute substantial amounts of phosphorus with 75-95 phosphates per tRNA and 4,700 phosphates per ribosome in rRNA (Sterner and Elser, 2002). Thus, the nucleic acid portion of the poly-na fraction likely contributed substantial amounts of phosphorus. Another contributor to phosphorus in the poly-na fraction could have been in the form of a phosphopolysaccharide, similar to teichoic acid, which has been seen in the bacteria *Lactobacillus kefiranofaciens* (Chojnacka et al., 2005; Mukai et al., 1992), that has structural functions in the cell wall, and contains approximately 5% P (Sterner and Elser, 2002). Polyphosphates, which are salts or esters of polyphosphoric acid (Sterner and Elser, 2002) would have also increased the phosphorus content of the poly-na fraction (Perry, 1976; Jensen, 1968; Chilvers et al., 1978). During times of high nutrients, algae can store excess phosphorus as polyphosphate (Chopin et al., 1997; Lafarga-De la Cruz et al., 2006; Omelon and Grynpas, 2008), either in linear (Darchambeau, 2005; Bental et al., 1988) or cyclic formation (Bental et al., 1988). Polyphosphates occur in most cell components, such as in the nucleus, mitochondria, endoplasmic reticulum, and cytoplasm (Werner et al., 2007; Omelon and Grynpas 2008). It is thought that polyphosphates will later be utilized during times of low available phosphorus (Omelon and Grynpas, 2008; Titelman et al., 2008), which was confirmed by Eixler et al. (2006). Eixler et al. (2006) demonstrated with the green algae *Chlorella vulgaris* that polyphosphate granules present at the onset of the experiment were not distinguishable while under phosphorus starvation, however, the granules became detectable once phosphorus was again supplied. Additionally, polyphosphates have also been established as an energy reserve in anoxic environments

(Omelon and Grynpas, 2008; Titelman et al., 2008) and have been shown to play a role in copper detoxification, as seen with *Skeletonema costatum* (Petersen and Kusk, 2000). Most polyphosphates, in terms of these experiments, would be found in the LMW compounds and polysaccharide fractions, with only a negligible amount being retained in the hot TCA insoluble fraction (i.e., protein on the GF/F filters) (Runcie and Larkum, 2001).

The LMW compounds fraction ranked third in the degree of ^{33}P incorporation into *R. salina*. The LMW compounds fraction consists of low molecular weight compounds (i.e., <1,000 daltons: Loh et al., 2004), including the phosphorus containing ATP (18% molecular phosphorus: Sterner and Elser, 2002). ATP is constantly being utilized as an energy source. When ATP is broken down, one phosphate group is cleaved and the other two remain attached to ribose (i.e., ADP). Conversely, when ATP is created from ADP, the two existing phosphate groups are joined by a new third group. Although external phosphorus concentrations were purposely kept low in both treatments, some water soluble orthophosphate in the cells may also have contributed to this fraction. Thus, all these highly labile and short-lived fractions should have resulted in high turnover in comparison to the other fractions, but this was clearly not the case in the algae. However, the copepods followed exactly this trend (Fig. 16b; Table 5), which will be discussed in sections to follow.

The lipid fraction incorporated the least amount of ^{33}P in both PI and PB cultured *R. salina*, an unexpected result as a large percentage of the important phospholipid pool of the algae should have ended up in this fraction. Phospholipids are found in the membranes of the endoplasmic reticulum, mitochondria, and chloroplasts (Sterner and

Elser, 2002). Regardless of its degree of incorporation, the lipid fraction demonstrates a 3.3 fold greater content in the PB cultured algae, further support that the nutrient regimes created algae that were biochemically different between the two treatments (Fig. 14c; Table 3). In their study with the plant, *Arabidopsis*, Frost et al. (2005) showed plants that were phosphorus starved had reduced levels of phospholipids, while levels of sulfolipids increased in their thylakoid membranes, which might account for the low ^{33}P incorporation into this fraction of the PI treatment. Thus, while the experimental nutrient media contained equal amounts of available phosphorus (Figs. 11a and 19a), the algae cultured on the PI media should have, in effect, been in a "P-limited" environment due to the excess of all other nutrients. This "P-limited", (i.e., PI), media may have caused a shift from more polar lipids (phospholipids) to more neutral lipids (wax esters), as it has been shown to occur in *Chlamydomonas reinhardtii* (Weers and Gulati, 1997) when cultured on media with a $1/100^{th}$ of its normal P concentration, resulting in higher ^{33}P values of the lipid fraction in PB cultured *R. salina* (Fig. 14c).

Steady state was reached in *R. salina* cells with regards to ^{33}P incorporation in all fractions and the total ^{33}P pool during the incorporation experiment (Fig. 14; Table 3). *R. salina* cells were fully labeled after 2-3 days, or 4-6 divisions (Wang et al., 1996). This degree of ^{33}P incorporation means that isotopic equilibrium (^{33}P:^{31}P) was achieved, well before the algae were presented to the copepods at 115 hours (Fig. 14). Copepods in the PI treatment were thus being presented with phosphorus depleted food (i.e., as seen in the totals – Fig. 14e), with all fractions of PI cultured algae except for the LMW compounds fraction being lower in phosphorus (Table 3).

Comparison in labeling pattern between percent ^{14}C and percent ^{33}P in various plankton and Rhodomonas salina

Many studies have been previously conducted on the biochemical incorporation pattern of ^{14}C into several types of phytoplankton and zooplankton (Table 19). In reviewing the literature, the protein fraction had the highest ^{14}C percent incorporation values and the polysaccharide fraction the lowest in the experiments that used protocols similar to the present one (Rivkin, 1985; Roman, 1991; Bochdansky et al., 1999; Thor et al., 2002). These results seem contradictory as polysaccharides tend to contain significant amounts of carbon (Morris et al., 1974), usually bound up in cellulose, hemi-cellulose and lignin (Anderson et al., 2004), and no phosphorus. It is thus possible that ^{14}C was insufficiently extracted into the polysaccharide fraction, using hot TCA, and some of it remained on the GF/F filter (i.e., protein fraction).

The biochemical fractionation protocol used in this thesis was based on protocols from several of the authors listed in Table 19. Rivkin (1985) employed much of the same protocol as the one discussed in this paper, however, there were differences in terms of volumes used. The current protocol extracted with only 2.5 ml of (1:2) chloroform/methanol and rinsed with a final 2 ml, while Rivkin (1985) extracted with the full 4.5 ml. Additionally, the current protocol used additional water and TCA for rinsing purposes, but the ratios of chloroform, methanol and water remained the same. Roman (1991) used a protocol based on Li et al. (1980), which was nearly identical to the one in the present paper except they extracted in hot TCA for only 30 minutes, while the current protocol extracted for one hour. The Bochdansky et al. (1999) protocol was a combination of Li et al. (1980) and Rivkin (1985), which in the end, was the basis for the

current protocol. Finally, the Thor et al. (2002) protocol diverged more than any other study in time, volume, and procedure. Thor et al. (2002) used a third of the extraction time in an ice bath and their TCA extraction time was half the current study's, and they used centrifugation for fractionation of all samples, while in the present study centrifugation was only used for phase separation of the LMW compounds and lipid fractions.

Even though two different elements were compared (i.e., ^{14}C: literature; ^{33}P: present study), there were several similarities between the percent incorporation patterns for ^{14}C and ^{33}P. The protein fraction contained the largest percentage regardless of isotope (Tables 4 and 19). At the third-largest, the LMW compounds fraction was identical for both isotopes with varying percentages (Tables 4 and 19). However, the isotopes diverged when the lipid and poly-na fractions were considered. The lipid fraction had the second highest percent incorporation in ^{14}C, while in ^{33}P the poly-na fraction was second. The switch in the relative dominance of C and P in these fractions seems plausible as the lipid fraction included general lipids, which contain significant amounts of carbon. However, even the presence of the P-rich phospholipids (~65% C; ~4.2% P; molecular percentages; Sterner and Elser, 2002) in the lipid fraction did not tip the balance. On the other hand, the poly-na fraction included the P-rich nucleic acids (~32.7% C; 8.7% P; molecular percentages; Sterner and Elser, 2002).

^{33}P allocation and overall distribution in Rhodomonas salina

During saturation, the average ^{33}P activity for every fraction, except LMW compounds, was significantly different between P-imbalanced (PI) and P-balanced (PB) cultured *Rhodomonas salina* (Table 3). ^{33}P allocation in the protein and in the poly-na

fractions was approximately 194% and168% higher in the PB treatment (Figs. 14a and 14d; Table 3), respectively. The lipid fraction showed the strongest response with 329% higher ^{33}P values PB treatment (Fig. 14c; Table 3). The totals were 186% higher in the PB cultured algae (Fig. 14e; Table 3). Expressed as a percentage of the total ^{33}P signal, however, ^{33}P allocation to the fractions was similar in both treatments (PI and PB) except for the lipid fraction (Table 4). Thus, the lipid pool appeared to be the most flexible, and interestingly, the LMW compounds the most homeostatically controlled pool. Given that all other nutrients were available in 10-fold higher concentration than P in the PI treatment, the large difference in the medium translated to only a ~2-fold difference in the algae in terms of total radioactivity. However, the cell-specific Chl *a* values were strikingly different between the two treatments (Figs. 12c and 20b), as well as carbon and nitrogen of *R. salina/Acartia tonsa* incorporation II (Figs. 20a and 20c).

An attempt was made to measure total particulate carbon using the wet-chemical technique based on Johnson (1949) and modified by Gosselin and Qian (1999) and Proft (1983), because the CHN analyzer and mass spectrometer were not to be contaminated with radioactive material. However, the attempts were unsuccessful because this method was, despite what literature claims (Johnson, 1949; Gosselin and Qian, 1999; Proft, 1983), unsuitable for biological material. Carbon samples of *R. salina* from *R. salina/A. tonsa* incorporation II were analyzed by mass spectrometry, as enough time had passed (ca. 2 years) so the instrument would not be contaminated with ^{33}P. The particulate carbon and nitrogen per cell values and associated statistics of *A. tonsa* incorporation II agreed with the increased Chl *a* per cell values. As the Chl *a* values in this experiment were similar to those of the *R. salina/A. tonsa* incorporation I, it is likely that similar

differences in particulate carbon and nitrogen would have been seen. As a result, much of the ^{33}P allocation seems to be under some homeostatic control in *R. salina*, which is quite different from some freshwater algae such as *Scenedesmus* that almost perfectly reflect the nutrient ratios of the environment (Sterner and Elser, 2002; Sterner et al., 2000; Sterner and Hessen, 1994).

Relative distribution of ^{33}P in Acartia tonsa

Acartia tonsa offered the P-balanced (PB) cultured *Rhodomonas salina* resulted in significantly greater incorporation rates of ^{33}P than those offered the P-imbalanced (PI) cultured *R. salina*. The rates ranged from 2 to 3-fold depending on the biochemical fraction (Table 5; Figs.16a-d). That the slopes of ^{33}P incorporation into *A. tonsa* were more different than steady state ^{33}P values in the algae demonstrated that biochemical changes in the *R. salina* occurred that were not apparent from the radioactivity alone.

The high incorporation rate of ^{33}P into the LMW compounds fraction of *A. tonsa* in the current study (Table 5; Fig.16b) was similar to the rapid turnover of P from metabolic pools such as ATP, indicated by Liu et al. (2006). Additionally, Correll and Tolbert (1962) found a high turnover rate for ATP for *Anabaena variabilis*. The poly-na fraction had similarly high turnovers, most likely because of the rapid turnover of nucleic acids (Table 5; Fig. 16d). This was confirmed by the second copepod incorporation experiment in which the RNA pool saturated quickly and had lower K_m values than the rest of the fractions (Fig. 22a). The protein and lipid fractions were slower to incorporate ^{33}P as demonstrated by their depressed incorporation rates (Table 5) and flatter slopes (Figs. 16a and 16c), consistent with the notion that these two fractions represent more structural compounds. Also, the lower incorporation of ^{33}P into the protein fraction of *A.*

tonsa was in agreement with the work of Roman (1991) with *A. tonsa* and *Thalassiosira weissflogii*, which demonstrated that proteins have low turnover in *A. tonsa*. The gradual turnover of protein in *A. tonsa* could have also been affected by the substantial amount of protein bound in the carapace. Exoskeletons of crustaceans consist of chitin (O'Brien et al., 1991), protein (Schwarz and Moussian, 2007; Raabe et al., 2005; Gagné et al., 2005; O'Brien et al., 1991), and calcium phosphate (Gagné et al., 2005) or hydroxyapatite (Vrede et al., 1999), which might have contributed phosphorus to this fraction if not completely dissolved by hot TCA, and would have thus remained on the filter with the "proteins". Lastly, the lipid fraction's low incorporation was in accordance with the fact that most of the phosphorus in this fraction should be in the form of phospholipids, which as part of the cell membranes are typically of structural (i.e., low turnover) nature.

While isotopic equilibrium was reached in the algae incorporation, it almost never occurred during the copepod incorporations. No fraction within the first copepod incorporation experiment became saturated; as a result, the uptake kinetics justified a linear model and allowed for the comparison of slopes of the ^{33}P uptake kinetics among fractions (Fig. 16; Table 5). The RNA (both treatments) and protein (PB treatment) fractions, on the other hand, of the second copepod incorporation experiment became saturated (Figs. 22a-b). The RNA fraction's turnover was notably faster than the protein fraction (170 vs. 230 hours). RNA is typically associated with protein production, which is likely to have a high turnover capacity. In the second incorporation experiment, the RNA fraction contained only pure RNA, while the protein fraction contained some DNA and phospholipids. It is thus unlikely that any other substances have contributed to the saturation kinetics observed in the RNA fraction. Overall, RNA has been shown to have a

rapid turnover time within *A. tonsa*, while protein/DNA/phospholipid turnover was not as immediate as in RNA (Fig. 22). This corroborates the concept that protein and DNA are often used as metrics of conservative (i.e., structural) cellular constituents. A longer experiment might be necessary for saturation to occur. Increasing the time-series would be a logical next step, especially considering the C:P and N:P ratios of *R. salina*, which were 370-380 and 41-61(Fig. 21; Table 10), respectively and the calanoid copepods' body C:P and N:P was only ~210 and ~26 (Sterner and Elser, 2002).

There are several explanations for the divergence in ^{33}P incorporation of the PI and PB fed *A. tonsa*, which can be categorized as nutrient-based or behavioral-based responses. Possible nutrient-based responses could be categorized as poor nutrient assimilation and increased filtration rate. The behavioral-based response could be categorized as reduced feeding on prey based on their "scent". Each category will be discussed separately.

The simplest explanation for differences in ^{33}P incorporation of the copepods stems from the nutrient quality of the algae, since quantity would not be a factor as they were fed *ad libitum* (i.e., at saturated levels). Given the differences in nutrient concentrations (Appendix IV), it was likely that the cells were chemically different. Chl *a* contains considerable amounts of carbon and nitrogen, $C_{55}H_{72}MgN_4O_5$ (Gossauer and Engel, 1996), but no phosphorus. *R. salina* from both *A. tonsa* ^{33}P incorporations exhibited a greater than 3 fold higher Chl *a* concentration in PI cultured *R. salina*. Cell-specific particulate carbon and nitrogen content demonstrated a clear difference between the PI and PB cultured *R. salina* (Figs. 20a and 20c). This contention is also supported by previous studies in which nutrient-limited cultured algae tend to have higher carbon

content than those of nutrient-sufficient algae (Skovgaard, 1996). Being fed at saturated levels, the copepods' assimilation rates were dictated by the amount of bulk food that could be ingested.

The reduced phosphorus incorporation presented here, a result of feeding on phosphorus-limited or deficient algae, is comparable to that seen by DeMott et al. (1998), who showed decreases in specific phosphorus content of *Daphnia* offered phosphorus-deficient green algae at high concentrations. This is despite compensatory feeding to make up the difference in lower relative phosphorus content (Anderson et al., 2004; Sterner and Hessen, 1994), which might have reduced the assimilation efficiency of non-phosphorus containing compounds (Boersma and Elser, 2006; DeMott et al., 1998) or an overall less efficient use of the nutrient of excess (Sterner and Hessen, 1994). The same concept has been demonstrated by the work of Darchambeau (2005) with *Daphnia* sp., which increased its filtration rate as the C: P of its food became greater than 320 in an attempt to acquire more P. Consequences of compensatory feeding include decreased growth, survival, and fecundity (Boersma and Elser, 2006) as a result of reduced P intake, while no significant energetic costs were likely to have been incurred (Kiørboe et al., 1985) since carbon is generally readily available.

Another possibility for the discrepancy in the ^{33}P uptake might have been that the algal cells modified their cell walls, based on the incoming nutrients. Increasing cell wall thickness has been shown to be a consequence of nutrient limitation, which ultimately reduced digestibility (van Donk et al., 1997). The change in cell morphology might have been the reason for lesser incorporation of ^{33}P into the PI fed copepods, as the study also showed phosphorus and nitrogen limited *Selenastrum* and *Chlamydomonas* migrated

through the gut of *Daphnia pulex* and *Daphnia magna* undigested (van Donk et al., 1997). As *A. tonsa* gut passage time is only 35-60 minutes (Arashkevich, 1977), increased wall thickness may reduce not only digestibility, but also assimilation, which could lead to physical impairments of the copepods as it has been documented in *Daphnia*. Sterner et al. (1993), in their work with *Scenedesmus acutus* and *Daphnia obtusa*, established that "sluggish" cladocerans with previous molts remaining attached to the animal were produced when reared on low phosphorus algae. This sluggishness and attachment of old molts would make the animals more susceptible to predation, thereby reducing chances of reproducing. Furthermore, Weers and Gulati (1997) found feeding juvenile *D. galeata* a diet of P-limited *C. reinhardtii* increased their maturation time and egg mortality, while reducing the number of offspring produced. It has yet to be determined if *R. salina* increases cell wall thickness in response to decreased nutrient availability, as they only have a periplast with an outer plasma membrane (Berge et al., 2008; Hibberd, 1971), which is typical of this genus (Lee, 1999) and class (Lichtle, 1979) of phytoplankton.

Lastly, *A. tonsa* could have been discouraged by the "scent" of the PI-cultured algae. *A. tonsa* chemoreceptors allow for pre-ingestion determination of food quality (Cowles et al., 1988). Calanoid copepods have been known to discriminate between foods of varying qualities (*Acartia clausi* and *Eurytemora herdmani*: Poulet and Marsot, 1978) and toxicities (*Acartia tonsa*: Colin and Dam, 2002; Selander et al., 2006).

Percent ^{33}P labeling pattern in Acartia tonsa

The percent ^{33}P incorporation for *Acartia tonsa* was, in descending order, LMW compounds, poly-na, protein and lipid (Fig. 18). The percentages of ^{33}P allocation were

equivalent between the PI and PB treatments (Table 7). The high percentage of ^{33}P (32-34%) in the LMW compounds fraction was most likely due to the uptake of digested monomers (simple sugars, amines, etc.) from *R. salina* by the gut tissue, which might not have been fully incorporated into the other fractions in the copepods and therefore still contributed to the LMW compounds fraction (Bochdansky et al., 1999; Roman, 1991). The percentage of ^{33}P in the protein fraction (19-21%) was rather high, perhaps deriving from the residual hydroxyapatite of the carapace (Hessen and Rukke, 2000; Vrede et al., 1999; Sunagawa, 2005). ^{33}P in the lipid fraction was higher (~14-17%) than that found for other marine calanoid copepods, as Ventura (2006) found the phosphorus pool of *Calanus finmarchicus* to consist of 10.7% phospholipids.

Comparison between ^{14}C and ^{33}P labeling patterns in Acartia tonsa

Several studies have examined the percent ^{14}C incorporation pattern in adult *Acartia tonsa* fed various algae (Table 19). Unlike the algae comparison, no common ^{14}C incorporation pattern emerged for *A. tonsa* (Table 19) and as such, it was difficult to determine meaningful relationships between percent ^{14}C and percent ^{33}P in *A. tonsa*. In three of four papers (Roman, 1991; Thor et al., 2002; Putland, 2005), two details became clear: (1) the lipid and poly-na fractions contained the least amount of label; (2) the protein and LMW compounds fraction the most (Table 19), whereas for ^{33}P the poly-na and LMW compounds fractions incorporated the most and the lipid and protein fractions the least. Generally values for each fraction were comparable in three out of four studies for 20-48%: protein, 20-40%: LMW compounds, 3-33%: lipid and 12-58%: poly-na (Table 19: Roman, 1991; Thor et al., 2002; Putland, 2005). Roman (1991) stated that most of the *Thalassiosira weissflogii* carbon taken up by *A. tonsa* initiates in the LMW

compounds fraction where it can be redirected to lipids, polysaccharides or protein synthesis. This redirection might also be applicable to ^{33}P and account for the elevated phosphorus in the LMW compounds fraction (Table 7).

Particulate phosphorus and chlorophyll a during the elimination experiment

The particulate phosphorus and Chl *a* values for non-radioactive *Rhodomonas salina* and *Thalassiosira weissflogii* provided to the copepods during the elimination experiments were presented volumetrically. *T. weissflogii* is a chain-forming diatom and as such, determination of cell differentiation and subsequent enumeration proved to be difficult. Since the aim of this particular experiment was elimination of radioactivity from the copepod, the cell specific values were less important because the algae came from the same cultures.

For the most part, the volume-specific particulate phosphorus and Chl *a* values for *R. salina* and *T. weissflogii* from the ^{33}P elimination experiment did not result in significantly different values between the designated P-imbalanced (PI) and P-balanced (PB) treatments within each food environment (Table 11). The chambers of each food environment were similar enough that any differences in elimination rates could be directly attributed to the copepods being pre-fed either the PI or PB cultured *R. salina*. However, in comparing the *R. salina* and *T. weissflogii* food environments, it became apparent that even though the chambers were set up to contain the same amount of carbon, by Chl *a* proxy (see Methods), different environments were created, with the *T. weissflogii* food environment containing more particulate phosphorus and Chl *a* (Table 12). There was a degree of settling in all algae food chambers, but *R. salina* appeared to

have settled much faster and to a greater degree than *T. weissflogii*, leaving less food available in the *R. salina* treatments.

^{33}P elimination from Acartia tonsa

Elimination of ^{33}P from *Acartia tonsa* occurred in both treatments and from all food environments. Feeding history (i.e., being pre-fed with P-imbalanced (PI) or P-balanced (PB) cultured *Rhodomonas salina*), had no effect on ^{33}P elimination rates from various biochemical fractions of *A. tonsa* in any food environment except for the lipid fraction in the ASW-P (no food) treatment (Figs. 24-25; Tables 13-14). The effect of feeding history, however, was only marginally significant (ANCOVA: df=23, F=5.27, p=0.03) and driven by a few data points at the end of the experiment (Figs. 24c and 25c; Table 14), so that not much confidence should be put into this result.

There was no difference in elimination rates among different biochemical fractions of either treatment or any food environment (Table 13 and 15). All fractions were found to release ^{33}P at the same rate. This result is in contrast to the slopes during ^{33}P uptake, where the LMW compounds and poly-na fractions had slopes significantly higher than those of protein and lipid, and RNA pools were quickly saturated (Figs. 16, 22 and 24-25; Tables 5 and 13). These data provide interesting insights into the kinetics of uptake and elimination. During uptake, freshly assimilated material accumulates in the labile LMW compounds and poly-na pools before it is fully incorporated into body tissue in the more structurally oriented protein and lipid pools. Once incorporated into body tissue, phosphorus is then lost or turned over at the same rate in all fractions. The latter is contrary to the initial hypothesis that the LMW compounds fraction would turnover more rapidly (see Introduction).

While slopes of ^{33}P elimination did not differ among feeding histories and biochemical fractions, striking differences in elimination rates among the different feeding treatments were observed. The slopes were steepest, and therefore elimination greatest, for the *Thalassiosira weissflogii* food environment, intermediate for the *R. salina* food environment, and lowest for the ASW-P food environment. Furthermore, assuming that ^{33}P elimination was representative for all phosphorus in the tissues, it took 4.2 to 4.4 days to lose 50% of total body phosphorus for the starving copepods (Table 17, ASW-P treatment). Uye and Matsuda (1988) reported a similar albeit slightly higher loss (i.e., 67%) in starving *Centropages abdominalis* over the similar period of time. By comparison, the biological half life of ^{33}P in humans is considerably longer (257 days: NCRP, 1980) than that of any population of *A. tonsa* with half lives of ~2-7 days.

Based on previous studies, the initial hypothesis was that *R. salina*, a very suitable prey for *A. tonsa* as it was raised on this alga for many generations, will be assimilated at a high rate, and thus cause a higher ^{33}P turnover and elimination rate. Støttrup and Jensen (1990) found that *R. salina* was nutritionally superior to *T. weissflogii* for *A. tonsa*, as well as the work of Tang (2005), which stated that *T. weissflogii* contained more indigestible material than *R. salina*. The hypothesis of faster elimination rates in the *R. salina* treatment than in the *T. weissflogii* treatment, however, was not only rejected but the opposite trend occurred. It may be tempting to attribute the difference between *T. weissflogii* and *R. salina* treatments to food quality, but one has to be cautious with this conclusion. As previously mentioned, during the experiment, *R. salina* cells settled relatively quickly on the bottom screens and were thus less available to *A. tonsa* feeding in the column. Thus, the *R. salina* treatment may have simply represented a lower food

concentration (Fig. 23; Table 11). *T. weissflogii*, on the other hand, settled to a lesser degree with a larger proportion of cells remaining suspended and available during the experiment (Fig. 23; Table 11). Experiments in the present study were performed using net cups instead of bottles on plankton wheels (see Bochdansky and Bollens, 2004; Selander et al., 2006; Tang et al., 2001). The reason for using net cups was to have fecal pellets and eggs settle through the net and thus avoid re-ingestion of ^{33}P through coprophagy (Lampitt et al., 1990) and egg cannibalism (Dagg, 1977).

In addition to faster settling rates, size differences between the algae may have contributed to the differences in prey and nutrient availability. *T. weissflogii* equivalent spherical diameter (ESD) ranges from 11.5-12.9 μm (Dam and Colin, 2005; Jones et al., 2002), while *R. salina* is smaller at 7.5-9 μm (Jakobsen, 2001; Berge et al., 2008). Calanoid copepods have elevated capture efficiencies for prey with an ESD greater than 10 μm (Becker et al., 2005). Adult *A. tonsa* prefer prey approximately 10-70 μm in size (Besiktepe and Dam, 2002; Berggreen et al., 1988). Thus, the copepods might have ingested *T. weissflogii* at a greater rate than the *R. salina* due to increased availability and capture efficiency. Larger cell size typically translates into increased nutrient content although nutrient content scales differently in diatoms than in cryptophytes (Strathman, 1969). *T. weissflogii* Chl *a* content is typically 4.0-6.2 pg Chl *a*/cell (Mayzaud et al., 1998; Sosik, 1988; Ruser et al., 1999), whereas *R. salina* values fall within a range of 0.4-1.7 pg Chl *a*/cell (see Discussion), with an average of about 1.0 pg Chl *a*/cell. These *R. salina* values are similar to the Chl *a* values presented here. The cell specific carbon value of 88 pg C/cell (Dam and Colin, 2005) for *T. weissflogii* was again higher than values for *R. salina*, which ranged from 21.8-65 pg C/cell (Ptacnik et al., 2004; Baines

and Fisher 2001; Tang et al., 2001; Broglio et al., 2003; Jeong et al., 2006; Chu et al., 2008), with an average of 42.9 pg C/cell. Cell specific phosphorus values were also different between the two algae. Average values were ~0.56 pg P/cell for *R. salina* (Fig. 12b), much lower than the 1.7 pg P/cell reported for *T. weissflogii* (Liu et al., 2006). Thus, *T. weissflogii* contains approximately 5 times the Chl *a*, 2 times the carbon (both near the range listed by literature) and ~3 times the phosphorus of *R. salina*. It is highly likely that settling of algae reduced encounter, ingestion, and assimilation rates of carbon and phosphorus and contributed to the shallower elimination slopes in the *R. salina* treatment. The observed differences are therefore primarily due to quantity of assimilated food, and to a lesser degree (if at all) due to food quality or digestibility. This means that phosphorus in the tissue was metabolized faster when incoming phosphorus was available than when the copepods were starving, supporting a very simple black-box model, i.e., one in which they may be treated as having equilibrated biochemical pools. It is important to note that no net losses of phosphorus from the body need to occur when the animals continue to feed; however, they do occur in starved animals.

Variation between percent ^{33}P content between Rhodomonas salina and Acartia tonsa

General average percent ^{33}P incorporation patterns varied between *Rhodomonas salina* and *Acartia tonsa*. In order of decreasing pool size, the incorporation pattern in *R. salina* was protein, poly-na, LMW compounds and lipid (Fig. 15; Table 4), while in *Acartia tonsa* the pattern was LMW compounds, poly-na, protein and lipid (Figs. 18 and 26a-c; Tables 7 and 18). The average percentages for poly-na were similar between *R. salina* (30.56-32.33%) and *A. tonsa* (29.59-32.02%). However, the average lipid percentages were approximately 1.5-2.8 times greater in *A. tonsa* compared to *R. salina*

(*R. salina*: 5.87(PI)-10.83 (PB) %; *A. tonsa*: 15.03-16.54%). Drastic differences in percent ^{33}P incorporations were seen in the protein and LMW compounds fractions. *R. salina* contained approximately 2.5 times more ^{33}P than *A. tonsa* in the protein fraction, while the percentage ^{33}P in the *A. tonsa* LMW compounds fraction was approximately 2.9 times greater (Tables 4, 7 and 18).

Boersma et al. (2008), Malzahn et al. (2007), and Sterner and Hessen (1994) indicated that there was a tendency for large variation of nutrient contents among autotrophs (typically non-homeostatic), while there was a lesser variation among herbivores (more homeostatic). Similarly, Sterner (1993) also found that at the plant-animal interface, a general discrepancy occurred between the biochemical makeup of *Daphnia obtusa* and *Scenedesmus acutus*. *Daphnia* was thus more homeostatic than *Scenedesmus*. However, based on the current study's percent ^{33}P incorporation, both *R. salina* and *A. tonsa* appear to regulate their nutrient intakes towards a more homeostatic nature (Figs. 15, 18 and 26). Both responded to the nutrient ratios of their resources and were thus not strictly homeostatic as defined by Sterner and Elser (2002). However, both organisms reduced the discrepancy in nutrient ratios of their nutrient sources (the inorganic medium for *R. salina*, and *R. salina* for *A. tonsa*). This homeostatic control of organisms is one of the important components that lead to the "emergent property" of the Redfield ratio and its relative constancy across ocean basins (Falkowski and Davis, 2004).

CONCLUSIONS

The experiments reported in this thesis provided new insights into the kinetics of phosphorus uptake in phytoplankton, and the kinetics of uptake and elimination of phosphorus in copepods. First, isotopic equilibrium was reached fairly quickly in all fractions in phytoplankton, whereas in the copepods ^{33}P incorporation remained in the linear uptake phase for the entirety of the incubations with the algae (ca. 5 days), with the exception of RNA and protein. With respect to the initial hypotheses, the conclusions are

a) *Rhodomonas salina*:

1) ^{33}P was incorporated at similar rates in all fractions and among treatments.

2) Surprisingly, incorporation of ^{33}P into the low molecular weight fraction, which included compounds <1,000 daltons, was not faster than into the other fractions.

3) Algae cultured on the P-balanced (PB) medium incorporated significantly more ^{33}P than the ones grown on a P-imbalanced (PI) medium although the same total amount of phosphorus was available to all.

In addition, the algae changed their biochemical makeup in response to elemental ratios in the medium as reflected by the variable Chl *a*, particulate carbon, and nitrogen contents of the cells. As all nutrient components (Chl *a*, particulate carbon, nitrogen, and phosphorus) had reached steady state by the conclusion of the ^{33}P incorporation time-series, it provided a suitable starting point for incorporation and elimination experiments with the copepods.

b) *Acartia tonsa*:

1) The ^{33}P incorporation rates were higher in the LMW compounds than in the protein, lipid, and poly-na fractions. Because nucleic acids dominated the phosphorus content of the poly-na fraction, incorporation into this fraction was also faster than into the protein and lipid fractions. Also, rapid saturation occurred in the RNA fraction, consistent with high turnover of this pool.

It is important to note, however, that the higher incorporation rates into the LMW fraction are not due to their higher turnover rates as initially suspected (see elimination experiments), but likely due to an accumulation of ^{33}P in a temporary pool that contained freshly assimilated material.

2) The P-balanced (PB) fed copepods incorporated ^{33}P faster than the P-imbalanced (PI) fed copepods in all fractions and in the totals.

3) In total, more phosphorus is incorporated into body tissue when fed the P-balanced (PB) cultured *R. salina* than when fed the P-imbalanced (PI) cultured *R. salina*. These results were a direct reflection of the lower phosphorus to biomass ratios of the algae.

The elimination of ^{33}P from the copepods followed the expected path of an exponential decay function. The conclusions, with regards to the original hypotheses, are:

1) The hypothesis that pre-feeding history (i.e., whether the *A. tonsa* fed on P-imbalanced (PI) or P-balanced (PB) cultured *R. salina*) plays a role in the elimination rates of ^{33}P was rejected for all fractions except for the lipid fraction, in which the result remained ambiguous.

2) Fed copepods (*R. salina* and *Thalassiosira weissflogii*) eliminated ^{33}P faster than starved (ASW-P) ones as new incoming nutrients replaced the resident ones.

In addition, the non-food treatment gave insight into the rate at which phosphorus was metabolized in starving *A. tonsa*. The effect that P-availability has on P-turnover is highly relevant as food availability fluctuates greatly in the natural environment.

3) The type of diet (*R. salina* and *T. weissflogii*) influenced the elimination rates of phosphorus. However, this observation was most likely due to reduced prey encounter rates of *R. salina* in comparison to *T. weissflogii*, and to a lesser extent due to a difference in nutrient content or digestibility.

4) All biochemical fractions displayed identical elimination rates. Since elimination rates are more representative for turnover rates (they are not influenced by temporary pools of freshly digested material, see above), one can conclude that no one phosphorus pool in the copepod tissues is more "labile" than the other.

The results of this study suggest that turnover rates of phosphorus in *A. tonsa* are primarily driven by the quantity of available prey phosphorus (cell number or phosphorus per other nutrient) and to a lesser extent by internal biochemical pools, food quality, or feeding history. The effect of food quality and digestibility of prey may still be factors that need to be explored in future studies on the dynamics of phosphorus in this important component of estuarine food webs. Consequently, and as a first approximation, treating *A. tonsa* as a "black box", with equilibrated biochemical pools, may thus be justified. The organisms in this study reduced the initial disequilibrium in nutrient ratios, ranging from 10-fold in the inorganic dissolved pool (Appendix IV), a 2-fold difference in algal ^{33}P radioactivity (Table 3), and a 2.5-fold difference in the copepods (Table 5 and Fig. 17). This dampening of variability in elemental nutrient levels in the inorganic world due to

non-strict homeostasis of organisms is at the very heart of the global stability of Redfield ratios.

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APPENDICES

Appendix I. Solution preparations for each trace element/macronutrient for M_7 (1-14) and the concentration in final M_7 medium (Kusk and Wollenberger 1999).

Stock solutions	Trace element/ Macronutrient	Concentration in stock (g/L)	Amount for M_7 (ml/L)	Concentration in final M_7 (µM)
1	**H_3BO_3**	14.2975	1.0	11.56
2	**$MnCl_2 * 4H_2O$**	1.80250	1.0	0.46
3	**LiCl**	1.53000	1.0	1.8
4	**RbCl**	0.355	1.0	0.21
5	**$SrCl * 6H_2O$**	0.760	1.0	0.14
6	**NaBr**	0.080	1.0	0.039
7	**$Na_2MoO_4 * 2H_2O$**	0.315	1.0	0.065
8	**$CuCl_2 * 2H_2O$**	0.08375	1.0	0.025
9	**$ZnCl_2$**	0.260	1.0	0.095
10	**$CoCl_2 * 6H_2O$**	0.200	1.0	0.042
11	**KI**	0.065	1.0	0.020
12	**Na_2SeO_3**	0.04380	1.0	0.013
13	**NH_4VO_3**	0.01150	1.0	0.0049
14	***Combined Fe-EDTA***			
	- $Na_2EDTA * 2H_2O$	2.5	5.00	1.68
	- $FeSO_4 * 7H_2O$	1.0		0.90

Appendix II. Solution preparations for trace element/macronutrient and combined vitamins for final M_7 medium (Kusk and Wollenberger 1999).

Final M_7	Trace element/ Macronutrient	Concentration in stock (g/L)	Amount for M_7 (ml/L)	Concentration in Final M_7 (mM)
(1-14)	***Combined trace elements***		50	
15	**$CaCl_2 * 2H_2O$**	29.380	10.00	1.9984
16	**$MgSO_4 * 7H_2O$**	24.660	5.00	0.6408
17	**KCl**	5.8	10	0.7780
18	**$NaHCO_3$**	64.8	1.00	0.7714
19	**$NaSiO_3 * 9H_2O$**	5.0	2.00	0.0383
20	**$NaNO_3$**	2.740	0.10	0.0032
21	**KH_2PO_4**	1.430	0.10	0.0011
22	**K_2HPO_4**	1.840	0.10	0.0017
23	***Combined Vitamins***		0.10	
	Thiamine hydrochloride	0.750		
	Cyanocobalamine	0.01		
	Biotine	0.0075		

Appendix III. Salts required per liter of seawater desired. *$NaHCO_3$ has different units (mg/L) and is added after a salinity of 30 was achieved to avoid precipitation (Kusk and Wollenberger 1999).

	Salt	Concentration (g/L)
1	**NaCl**	70.1
2	**Na_2SO_4**	11.7
3	**KCl**	2.030
4	**KBr**	0.293
5	**$Na_2B_4O7 * 10H_2O$**	0.113
6	**$MgCl_2 * 6H_2O$**	31.7
7	**$CaCl_2 * 6H_2O$**	6.6
8	**$SrCl_2 * 6H_2O$**	0.066
9	**$NaHCO_3$**	2.83*

Appendix IV. Preparation of f/2, f/2-Si modified (PI) and f/20-Si (PB) from Guillard and Ryther (1962) and Guillard (1975). The f/2-Si medium consists of the same volume and concentrations as standard f/2, but with no addition of $NaSiO_3$.

Compound	Stock Solution (g/L dH_20)	Volume in 1 L f/2	Concentration in final f/2 medium (µM)	Volume in 1 L f/2-Si modified	Concentration in final f/2-Si modified medium (µM)	Volume in 1 L f/20-Si modified	Concentration in final f/20-Si modified medium (µM)
$NaNO_3$	75.0	1.0	883	1.0	883	0.1	88.3
NaH_2PO_4	5.0	1.0	36.3	0.1	3.63	0.1	3.63
$NaSiO_3$	30.0	1.0	107	N/A	N/A	N/A	N/A
Trace Metals	Appendix V	1.0	See Appendix V	1.0	See Appendix V	0.1	See Appendix V
Vitamins	Appendix VI	0.5	See Appendix VI	0.5	See Appendix VI	0.05	See Appendix VI

Appendix V. Preparations of trace metal solutions for f/2 medium from Guillard and Ryther (1962) and Guillard (1975).

Compound	Quantity	Stock Solution (g/L dH_20)	Concentration in final medium µM
$FeCl_3 * 6H_2O$	3.15 g	-	1×10^{-2}
$Na_2EDTA * 2H_2O$	4.36 g	-	1×10^{-2}
$CuSO_4 * 5H_2O$	1.0 ml	9.8	4×10^{-5}
$Na_2MoO_4 * 2H_2O$	1.0 ml	6.3	3×10^{-5}
$ZnSO_4 * 7H_2O$	1.0 ml	22.0	8×10^{-5}
$CoCl_2 * 6H_2O$	1.0 ml	10.0	5×10^{-5}
$MnCl_2 * 4H_2O$	1.0 ml	180.0	9×10^{-4}

Appendix VI. Preparations of vitamin solution for f/2 medium from Guillard and Ryther (1962) and Guillard (1975).

Compound	Stock Solution (g/L dH_2O)	Quantity	Concentration in final f/2 medium µM
Vitamin B_{12}	1.0	1.0 ml	1.0×10^{-7}
Biotin	0.1	10 ml	2.0×10^{-6}
Thiamine * HCl	-	200 mg	3.0×10^{-4}

Appendix VII. Preparations of solutions for particulate phosphorus determination from Solorzano and Sharp (1980).

Compound	Stock Solution	Concentration
Na_2SO_4	12 g/ 500 ml dH_2O	0.17 M
$MgSO_4$	2 g/ 1000 ml dH_2O	0.017 M
HCL	16 ml/ 1000 ml dH_2O	0.2 M

Appendix VIII. Preparations for mixed reagents, standards and blanks for particulate phosphorus determination from Strickland and Parsons et al. (1984)

Compound	Stock Solution	Volume (ml) per 8 samples
$(NH_4)_6Mo_7O_{24} * 4H_2O$	15 g/500 ml dH_2O	2
H_2SO_4	140 ml/900 ml dH_2O	5
$C_6H_8O_6$	27 g/500 ml dH_2O	2
$C_4H_4KO_7$	0.34 g/250 ml dH_2O	1
Phosphorus standard (KH_2PO_4)	0.816 g/1000 ml dH_2O	N/A
Dilute phosphorus standard	10 ml Standard + 1 ml of chloroform/ 1000 ml dH_2O	N/A
Sample phosphorus standard	0.5 Dilute Standard / 9.5 ml dH_2O	N/A
Sample Blank	10 ml of dH_2O	N/A

Appendix IX. Preparations of solutions for RNA extraction (Sambrook and Russell 2001).

	Compound	Stock solution	Concentrations in solution
RNA precipitation solution			
	NaCl	1.74 g/ 25ml	1.2 M
	$C_6H_6Na_2O_7*1.5H_2O$	5.3 g/ 25 ml	0.8 M
Buffer	TE	1 ml made up to 20 ml with dH_2O	

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