13 Phosphorus Cycle in Seawater: Dissolved and Particulate Pool Inventories and Selected Phosphorus Fluxes

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GENERAL INTRODUCTION

Phosphorus (P) is an essential macronutrient for all living organisms; life is truly built around P (deDuve, 1991). In the sea, P exists in both dissolved and particulate pools with inorganic as well as organic origins. The uptake, remineralization/hydrolysis and exchanges (by both physical and biological processes) of these various pools are the essential components of the marine P cycle (Figure 13.1). Compared to the much more extensive investigations of carbon (C) and nitrogen (N) dynamics in the sea, P pool inventories and fluxes are less well documented although no less important.

Herein, we present the principle and stepwise procedures for the accurate estimation of (1) dissolved orthophosphate (HPO_4^{2-}; hereafter referred to as *Pi*), soluble reactive P (SRP), total dissolved P (TDP) and particulate P (PP) as minimal constraints on P pool inventories, (2) dissolved and particulate adenosine-5′-triphosphate concentrations (D-ATP and P-ATP, respectively), (3) *Pi* uptake/regeneration and dissolved organic P (DOP) production rates using radiolabeled *Pi* (^{32}P or ^{33}P) precursors and (4) turnover rate estimation of the α-P and γ-P of intracellular ATP. The latter

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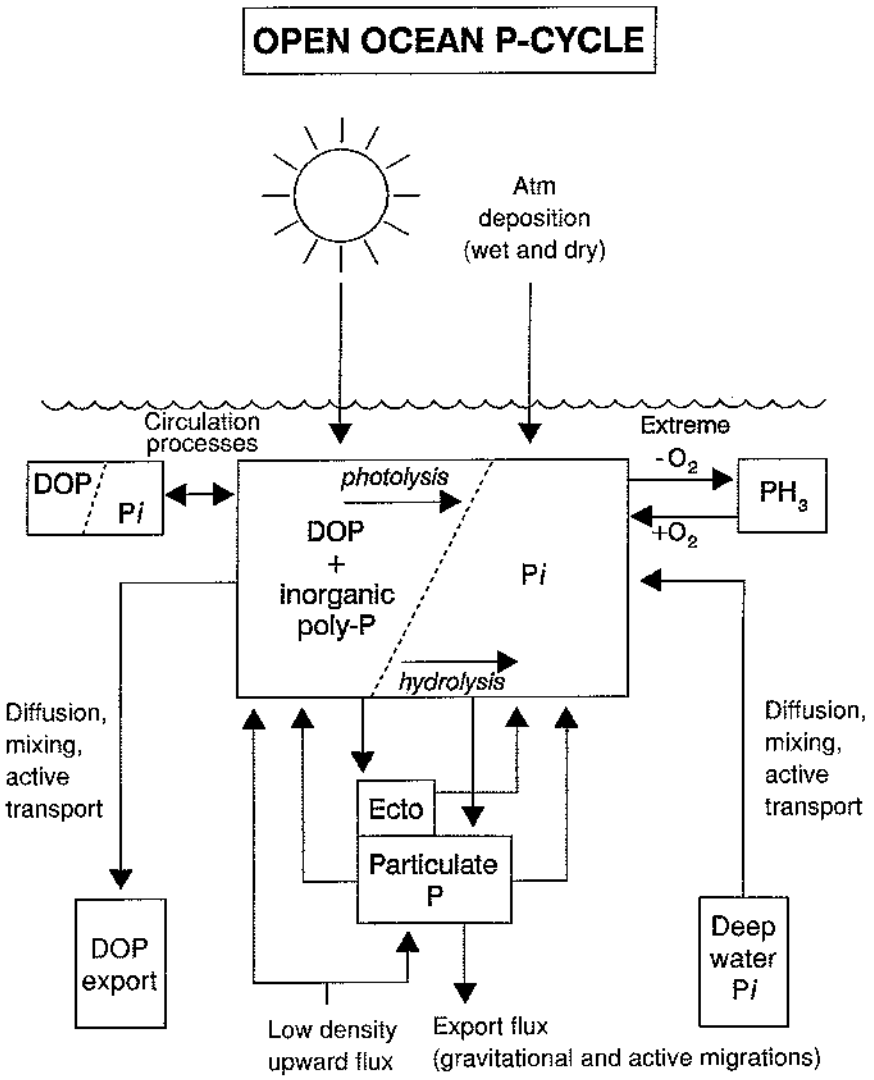


Figure 13.1. Schematic representation of the open ocean P-cycle showing the various sources and sinks of inorganic and organic P pools, including biotic and abiotic interconversions. The large rectangle in the center represents the upper water column TDP pool comprised of P*i*, inorganic polyphosphate and a broad spectrum of largely uncharacterized DOP. Ectoenzymatic activity (Ecto) is critical for microbial assimilation of selected TDP compounds. Particulate P, which includes all viable microorganisms, sustains the P-cycle by assimilating and regenerating P*i*, producing and hydrolyzing selected non-P*i* P, especially DOP compounds, and by supporting net particulate matter production and export. Atmospheric deposition, horizontal transport and the upward flux of low density organic P compounds are generally poorly constrained processes in most marine habitats. Phosphine (PH_3), shown at the right, is the most reduced form of P in the biosphere and is generally negligible except under very unusual, highly reduced conditions.

measurements provide information on microbial growth rate, total energy flux and the biologically available P (BAP) pool. While this by no means represents a comprehensive investigation of P cycle processes (see Table 13.1), these methods collectively provide a protocol suite that is suitable for an initial study of P dynamics in selected marine habitats.

◆◆◆◆◆◆ DETECTION OF PHOSPHORUS AND P-CONTAINING COMPOUNDS IN SEAWATER

The analysis of dissolved and particulate P-containing compounds in seawater is neither simple nor straightforward. Strickland and Parsons (1972) defined eight different operational classes of P compounds based on reactivity with the acidic molybdate reagents, ease of hydrolysis and particle size. These range from 'inorganic, soluble and reactive,' presumably *Pi*, through 'enzyme hydrolyzable phosphate' (*Pi* released following treatment with the enzyme alkaline phosphomonoesterase), to 'inorganic, particulate and unreactive' (presumably P-containing minerals). Some of the operationally-defined pools have no convenient analytical method of determination while others can be estimated only as the difference between two operational classes with partially overlapping specificity. Only a very few specific compounds or compound classes can be readily detected at the low concentrations typically found in seawater (Table 13.1).

There is likely to be a broad spectrum of P-containing compounds in seawater. Our inability to completely characterize these various dissolved and particulate pools currently limits further progress towards a comprehensive understanding of the marine P cycle. Even the most routine analytical method employed for *Pi* concentration measurement in seawater fails to provide an accurate estimate, especially for habitats where the DOP pool exceeds the dissolved inorganic P pool (e.g. most subtropical and tropical surface waters).

Quantitative analyses of P in seawater have traditionally relied upon the formation of a 12-molybdophosphoric acid (12-MPA) complex and its subsequent reduction to yield a highly colored blue solution, the extinction of which is measured by absorption spectrophotometry (Fiske and Subbarow, 1925; Murphy and Riley, 1962). Over the years, numerous improvements have been introduced to the basic method so that substantial variability now exists in the conditions used for color development, final reduction of the 12-MPA complex, and the treatment of potentially interfering compounds.

Although the stepwise chemical procedures for P determination are straightforward and fully amenable to automated analysis, there are many complexities, both analytical and conceptual, inherent in measuring and interpreting P concentrations in seawater (Tarapchak, 1983). For example, the soluble reactive P (SRP) pool measured by the standard Murphy–Riley procedure is not necessarily equivalent to the concentration of *Pi*, but may also include DOP compounds that are hydrolyzed

Table 13.1 The marine P-cycle: methods for selected inventories and fluxes[1]

Parameter	Method and comments	Reference
Inventories: dissolved pools (nmol P l^{-1})		
SRP (standard)	Molybdenum blue spectrophotometry	Murphy and Riley, 1962
SRP (6 sec assay)	Low acidity, rapid molybdenum blue color development; minimal hydrolysis of DOP	Chamberlain and Shapiro, 1969
SRP (extraction)	Isobutanol extraction of molybdenum blue dye to enhance assay sensitivity	Stephens, 1963
SRP (malachite green)	Use of the dye malachite green oxalate to enhance sensitivity	Fernández *et al.*, 1985
SRP (long path length)	Use of long capillary cell detector for high sensitivity (1 nM) P*i* detection	Ormaza-González and Statham, 1991
P*i* (MAGIC)	MAGIC/molybdenum blue spectrophotometry; high sensitivity, high specificity	Karl and Tien, 1992; Thomson-Bulldis and Karl, 1998
P*i* (enzymatic)	Measurement of competitive inhibition of *E. coli* alkaline phosphatase by ambient P*i*	Pettersson, 1979
ATP and GTP	MAGIC/firefly bioluminescence	Björkman and Karl, 2000
ATP	Charcoal binding-elution/firefly bioluminescence	Azam and Hodson, 1977
c-AMP	Charcoal binding-elution, radioimmuno-assay; seawater and sediments	Ammerman and Azam, 1981
Vitamin B_{12}	$^{14}CO_2$ uptake rate by a B_{12}-starved culture of *Cyclotella nana*	Carlucci and Silbernagel, 1966
DNA	Ethanol precipitation/Hoechst-33258 fluorometry	DeFlaun *et al.*, 1986
DNA and RNA	CTAB precipitation/dye binding	Karl and Bailiff, 1989
Phosphomonoesters	P*i* concentration increase following incubation with alkaline phosphatase	Francko, 1984
Phosphomonoesters	Selective low-pressure (110 W) UV-photodecomposition followed by P*i* (MAGIC)	Karl and Yanagi, 1997
Alkaline phosphatase-hydrolyzable P	Automated analysis by enzymatic flow injection using immobilized *E. coli* alkaline phosphatase, measurement of P*i*	Shan *et al.*, 1994

Inorganic and organic polyphosphate	TDP difference between UV photo-oxidation (which does not recover polyphosphates) and acid digestion methods	Solórzano and Strickland, 1968
Phosphonates	Tangential flow concentration/^{31}P-NMR	Clark *et al.*, 1999
Phosphonates	Combined enzymatic-acid hydrolysis and a separate nitrate oxidation treatment; phosphonate-P is determined by difference	Cembella and Antia, 1986
BAP (Rigler)	Measure uptake rate of simultaneous additions of ^{31}P*i* and ^{32}P*i* by selected algae under near *in situ* conditions, followed by kinetic analysis of the data to provide an upper constraint on BAP	Rigler, 1966; Brown *et al.*, 1978 and, especially, Tarapchak and Herche, 1988
BAP (bioassay)	Use of P-starved algal growth to estimate total bioavailable P	Chamberlain and Shapiro, 1969
BAP (ATP labeling)	Addition of ^{32}P*i* (^{33}P*i*) and determination of ^{32}P*i* (^{33}P*i*)/^{31}P of γ-P of ATP at isotopic equilibration to estimate total bioavailable P	Karl and Bossard, 1985b
Non-P*i* phosphorus	Pre-treatment of water sample to remove P*i*, persulfate oxidation of non-P*i* compounds to P*i* followed by MAGIC	Thomson-Bulldis and Karl, 1998
TDP (persulfate)	Autoclave, acid-persulfate treatment of water sample, measure P*i*; this method does not quantitatively recover phosphonates	Menzel and Corwin, 1965
TDP (UV)	UV-H_2O_2 assisted photo-oxidation of combined P, measure P*i*; this method does not quantitatively recover polyphosphates or selected phosphonates	Armstrong *et al.*, 1966
TDP (UV + persulfate)	Sequential UV and persulfate (see above) treatments	Ridal and Moore, 1990
TDP ($MgSO_4$)	Mix seawater sample with $MgSO_4$, evaporate to dryness, react at 450-500°C for 2 h, add HCl, heat at 80°C, measure P*i*	Solórzano and Sharp, 1980

Table 13.1 continued

Parameter	Method and comments	Reference
TDP (nitrate oxidation)	Mix seawater sample with $Mg(NO_3)_2$, evaporate to dryness under high temperature, add HCl and heat at 60°C, measure P*i*; of the TDP methods available, this is the most efficient for the hydrolysis of recalcitrant compounds such as phosphonates and polyphosphates	Cembella *et al.*, 1986
Inventories: particulate pools (nmol P l^{-1})		
ATP, ADP, AMP	Tris buffer extraction of cells, firefly bioluminescence; enzymatic phosphorylation of ADP and AMP to ATP, firefly bioluminescence	Karl and Holm-Hansen, 1978
GTP, GDP, GMP	Tris buffer extraction of cells, firefly bioluminescence; enzymatic phosphorylation of GDP and GMP to GTP, firefly bioluminescence	Karl, 1978a,b
DNA	Collect particulate matter, react with 3,5-diaminobenzoic acid (DABA), measure fluorescence (many other fluorescent dyes can also be used including DAPI and Hoechst-33258)	Holm-Hansen *et al.*, 1968
RNA	Collect particulate matter, purify RNA fraction by chemical extraction procedures, react with orcinol, measure via spectrophotometry	Lin and Schjeide, 1969
Total nucleic acid (double stranded)	Chemical extraction and partial purification of RNA and DNA from particulate matter, followed by detection using ethidium bromide-induced fluorescence	Sakano and Kamatani, 1992
Phosphonates	NaOH extraction, solid-state, Magic-angle spinning ^{31}P nuclear magnetic resonance (MAS-NMR); applied to marine sediments	Ingall *et al.*, 1990

Phospholipid	Chloroform-methanol lipid extraction, acid hydrolysis, measure P*i*	White *et al.*, 1979; Findlay *et al.*, 1989
Inositol isomers	Gas chromatographic – mass spectrometric analysis; applied to marine sediments	White and Miller, 1976
Acid soluble organic-P, phospholipids, oligopolyanions and RNA-P	Sequential extraction/separation of total P into compound classes; measure P*i* in isolated compound classes	Correll, 1965
Total P	Collect particulate matter, combust 450°C, add HCl, heat at 60°C, measure P*i*	Karl *et al.*, 1991
Total P	Collect particulate matter onto a GF/F filter, add $MgSO_4$, react at 450–500°C for 2 h, add HCl, heat at 80°C, measure P*i*	Solórzano and Sharp, 1980

Physiological potentials (nmol P or μg substrate l^{-1} h^{-1})

Alkaline phosphatase activity (EC 3.1.3.1)	Hydrolysis of glycerol-P and measurement P*i* released	Taga and Kobori, 1978
Alkaline phosphatase activity (EC 3.1.3.1)	Hydrolysis of P-nitrophenyl phosphate and measurement of nitrophenol released	Kuenzler and Perras, 1965
Alkaline phosphatase activity (EC 3.1.3.1)	Hydrolysis of non-fluorescent methylumbelliferyl phosphate (MUFP) to highly fluorescent MUF	Hoppe, 1993
Alkaline phosphatase activity (EC 3.1.3.1)	Hydrolysis of non-fluorescent 3-0 methyl fluorescein phosphate to highly fluorescent derivative	Perry, 1972
5′-Nucleotidase activity (EC 3.1.3.5)	Timed release of ^{32}P from exogenous [γ-^{32}P]-ATP	Ammerman and Azam, 1985
Deoxyribonuclease activity (EC 3.1.4.5)	Measurement of deoxyribose production rate, by DABA-induced fluorescence, following addition of DNA to sample preparation (analogous technique can also be used for RNase activity)	Maeda and Taga, 1973
Substrate bioavailability	^{32}P*i* uptake rates in presence and absence of test compound, relative to equimolar additions of ^{31}P*i*	Björkman and Karl, 1994

Table 13.1 continued

Parameter	Method and comments	Reference
Fluxes (dissolved and particulate)		
Pi uptake/regeneration	^{32}Pi (^{33}Pi) addition, *in situ* incubation and timed measurements of specific radioactivity (radioactivity/mass)	Harrison, 1983
D-DNA production	Bacterial [^{3}H]-thymidine incorporation into D-DNA	Paul *et al.*, 1987
D-DNA uptake	Addition of [^{3}H]-DNA (*E. coli*) to seawater, follow fate	Paul *et al.*, 1987
DOP production and turnover	^{32}Pi (^{33}Pi) addition, *in situ* incubation, measurement of initial specific radioactivity and post-incubation isolation of $DO^{32}P$ ($DO^{33}P$) from precursor ^{32}Pi (^{33}Pi)	Orrett and Karl, 1987; Björkman *et al.*, 2000
Dissolved P regeneration rate	Pulsed ^{32}Pi (or ^{33}Pi) uptake followed by ^{31}Pi chase and measurement of post-chase accumulation rate of dissolved ^{32}P (or ^{33}P)	Hudson and Taylor, 1996
P-DNA production (bacterial)	[^{3}H]-thymidine incorporation into purified DNA	Fuhrman and Azam, 1980
P-RNA/P-DNA production (microbial)	[^{3}H]-adenine incorporation into purified RNA and DNA fractions, determination of mass flux from precursor specific radioactivity estimation	Karl, 1981
P-RNA/P-DNA production (microbial)	As above for [^{3}H]-adenine incorporation, except using ^{32}Pi (^{33}Pi) radiotracers	Karl and Bossard, 1985b
Phospholipid synthesis	Incubate sample with ^{32}Pi (or ^{33}Pi), isolate phospholipid fraction, radioassay	White *et al.*, 1977
Growth rate and energy flux (ATP turnover)	Time course incubation of water sample with ^{32}Pi (or ^{33}Pi), extract and purify ^{32}P-ATP, measure α-P and γ-P specific radioactivity of ATP pool	Karl, 1993
PP export	Sediment trap particulate matter collections, measurement of total P	Knauer *et al.*, 1979; Karl *et al.*, 1996

[1] Abbreviations: SRP — soluble reactive P; *Pi* — orthosphosphate ion; DOP — dissolved organic P; TDP — total dissolved P; BAP — biologically available P; MAGIC — magnesium-induced co-precipitation; ATP — adenosine-5′-triphosphate; GTP — guanosine-5′-triphosphate; c-AMP — 3,5 cyclic adenosine monophosphate; DNA — deoxyribonucleic acid; RNA — ribonucleic acid; NMR — nucleic magnetic resonance, UV — ultraviolet light

under the acidic reaction conditions. Significant differences between SRP and P*i* have been observed for nearly every natural aquatic ecosystem where more rigorous and specific methods of P*i* analysis have been employed (Kuenzler and Ketchum, 1962; Jones and Spencer, 1963; Rigler, 1968; Pettersson, 1979; Karl and Tien, 1997; Thomson-Bulldis and Karl, 1998), and even different SRP methods return unequal estimates of '*Pi*' when employed with common seawater samples (Karl and Tien, 1997). As discussed below, accurate determination of P*i* is absolutely essential for the application of ^{32}P*i* or ^{33}P*i* tracer studies if mass flux estimation (P*i* uptake or P*i* regeneration rates) is the experimental objective.

Most investigators measure both SRP and TDP; the latter is also quantified by the molybdenum blue reaction following hydrolysis (Table 13.1). Only the nitrate oxidation method provides a quantitative recovery of total P, including polyphosphates and phosphonates (Cembella *et al.*, 1986). Monaghan and Ruttenberg (1999) have recently re-evaluated the efficacy of several methods for TDP estimation in coastal waters, and recommend the Solórzano and Sharp (1980) procedure. It is conceivable, even likely, that the behavior of these various methods is site-specific and that the efficiency of soluble non-reactive P (SNP) hydrolysis to SRP is dependent upon the chemical composition of the P pool in a given habitat.

◆◆◆◆◆◆ HIGH-SENSITIVITY, HIGH-SPECIFICITY ASSAY FOR P*i* AND MEASUREMENTS OF TDP

Principle

In most oligotrophic oceanic environments, P*i* determinations are unreliable because the ambient concentrations approach the limit of analytical detection (~20–40 nM) for the standard Murphy–Riley method. At these low concentrations, the variability of replicate determinations can be ±25%, or greater. Consequently, several modifications and new analytical procedures have been introduced to improve the detection limit and precision of environmental P*i* analyses (Table 13.1).

The *mag*nesium-*i*nduced *co*precipitation (MAGIC) method for the precise determination of P*i*, described by Karl and colleagues (Karl and Tien, 1992; Thomson-Bulldis and Karl, 1998) can be used to eliminate interference from non-P*i*, P-containing compounds thereby providing a more accurate estimate of P*i* that is necessary for reliable P-cycle flux determinations (Table 13.1). The method relies on the quantitative removal of P from solution by *in vitro* formation of brucite [$Mg(OH)_2$], initiated by the addition of NaOH. The precipitate is collected by centrifugation and dissolved in HCl for P*i* determination by the standard Murphy–Riley molybdenum blue reaction. This MAGIC procedure can be used to routinely effect a P*i* pre-concentration ranging from 2–100-fold, thereby providing a method which can reliably detect subnanomolar concentrations in seawater. The method is highly reproducible; typical estimates of precision for triplicate SRP determinations in the 10–100 nM

range are from 1 to 3%. The method is versatile and has many potential ecological applications.

Pi Determination by the modified MAGIC technique

Equipment and reagents

- Acid cleaned high-density polyethylene (HDPE) sampling bottles
- Polypropylene centrifuge tubes, 50 ml (Corning)
- Plastic 50 ml pipettes for sample dispensing
- Automatic pipettes or repipetter for reagent dispensing
- Centrifuge with capacity to hold 50 ml tubes
- Spectrophotometer, Beckman DU 640, or equivalent, and cuvette cells (10 cm)
- Sodium hydroxide 1 M (NaOH, Fluka Biochemica #71689)
- Hydrochloric acid 0.1 M (Fisher trace metal grade HCl #A508-212)
- Arsenate reducing mixture:
  1. Sulfuric acid 3.5 N (H_2SO_4 Fisher #A300-212)
  2. Sodium metabisulfite 14% w/v ($Na_2O_5S_2$, Fluka Biochemica #71930)
  3. Sodium thiosulfate 1.4% w/v ($Na_2O_3S_2$, Fluka Biochemica #72049)

  All solutions are made up in distilled, deionized water (DDW). Sodium metabisulfite must be freshly prepared. Mix reagents 1 and 2, add 3 in the volumetric proportions 2:4:4.
- Primary standard potassium phosphate (KH_2PO_4); prepare dilution series 0.015-0.5 µM, including blank
- Certified reference material (CSK-nutrient element, Wako Chemicals #27874099)
- Molybdenum blue reaction mixture:
  1. Sulfuric acid 5 N (H_2SO_4, Fisher #A300-212)
  2. Ammonium molybdate 30% w/v ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, Fisher #A674-500)
  3. Ascorbic acid 5.4% w/v (Fisher #A61-100)
  4. Potassium antimonyl tartrate 0.136% w/v ($KSbC_4H_4O_7 \cdot 0.5\ H_2O$, Mallinckrodt #2388)

  All solutions are made up in DDW. Ascorbic acid must be freshly prepared, or store frozen prior to use. All other solutions are stable for months, or longer. Mix reagents 1 and 2, add 3 and 4 in the volumetric proportions 5:2:2:1.

Assay

1. The stored, frozen samples are thawed in a water bath to room temperature.
2. Triplicate 50 ml sample aliquots are placed into new 50 ml polypropylene centrifuge tubes using a 50 ml plastic pipette. Work from anticipated low to high concentrations (i.e. from surface to deep) or thoroughly rinse pipette in distilled water between samples.

3. Add 150 µl 1 M NaOH to each 50 ml aliquot (0.3%, v/v).
4. Cap tubes tightly, and invert to mix the floc produced by the NaOH addition.
5. Centrifuge at 1000g at room temperature for 60 min.
6. Aspirate the supernatant using a clean Pasteur pipette attached to a vacuum line and collection flask. Take care not to disturb the small pellet.
7. For a routine five-fold sample concentration, add 10 ml of 0.01 M HCl to each tube and vortex to dissolve the pellet. Greater concentration factors up to and exceeding 100-fold can be effected by decreasing the final volume of HCl, by increasing the initial seawater volume, by increasing the acidity of the HCl, or all three.
8. Add 1 ml reduction mix to each 10 ml sample. Vortex and allow to react for 15±1 min.
9. After 15±1 min add 1 ml molybdenum blue reaction mix to each 10 ml sample and vortex to mix. Wait at least 15 min at room temperature to ensure full color development.

Note: Standards are prepared in surface seawater and treated the same as the samples. Blank samples are prepared from phosphate-free seawater (i.e. supernatant from MAGIC treated samples). Samples and standards are read at 880 nm in a 10 cm cell.

Optional: Tracer addition of ^{32}Pi (or ^{33}Pi) added to the original sample prior to NaOH addition can be used to precisely determine the recovery of *Pi* for each subsample. While this is not necessary for routine use, it does provide the most accurate and precise estimation of *Pi*.

The measurement of TDP is also operationally-defined; typically a high-intensity UV photooxidation (Armstrong *et al.*, 1966) or high temperature wet persulfate oxidation (Menzel and Corwin, 1965) or a combined procedure (Ridal and Moore, 1990) pre-treatment is used to convert SNP to *Pi* for subsequent analysis by the standard molybdenum blue assay. However, it is well known that certain P-containing compounds (e.g. inorganic polyphosphates, nucleotide di- and triphosphates) are not quantitatively recovered by standard UV photooxidation procedures; neither method quantitatively recovers P from phosphonate compounds. Depending upon the methods used, the difference between the measurement of TDP and either *Pi* or SRP will be termed SNP (i.e. SNP = [TDP]-[SRP]) or non-*Pi* P (N-*Pi* P = [TDP]-[*Pi*]), where SNP ≠ non-*Pi* P (Thomson-Bulldis and Karl, 1998). Furthermore, the MAGIC technique provides the opportunity for a direct rather than an indirect measurement of SNP (technically, non-*Pi* P) by an initial quantitative removal of *Pi*, followed by sample hydrolysis-oxidation and *Pi* determination (Thomson-Bulldis and Karl, 1998). This is especially useful for measure-

ments of non-*Pi* P in waters where *Pi* is the dominant form, for example in the deep sea. As emphasized previously, there is no *a priori* relationship between these operationally-defined pools and the more ecologically-relevant BAP pool.

TDP determination by persulfate oxidation and MAGIC

Equipment and reagents

- Acid-cleaned high-density polyethylene (HDPE) sampling bottles
- Acid-cleaned autoclavable glass centrifuge tubes 50 ml with Teflon lined caps (Kimble)
- Plastic 50 ml pipettes for sample dispensing
- Automatic pipettes or repipetter for reagent dispensing
- Centrifuge with capacity to hold 50 ml tubes
- Autoclave
- Sodium hydroxide 1 M (NaOH, Fluka Biochemica #71689)
- Hydrochloric acid 0.1 M (Fisher trace metal grade HCl #A508-212)
- Potassium persulfate 5% w/v ($K_2S_2O_8$, Fisher #P281)
- Primary standard potassium phosphate (KH_2PO_4); prepare dilution series 0.015–0.5 µM, including blank
- Spectrophotometer Beckman DU 640, or equivalent, and cuvette cells (10 cm)
- Certified reference material, arsenate reducing mixture and molybdenum blue reagents (all as above)

Assay

1. The stored, frozen samples are thawed in a water bath to room temperature. Typically, subsamples for TDP analysis are processed along with *Pi* determinations (see above).
2. Triplicate 40 ml sample aliquots are placed into 50 ml acid-washed glass centrifuge tubes using a 50 ml plastic pipette. Work from anticipated low to high concentrations (i.e. from surface to deep).
3. Add 6.4 ml potassium persulfate solution to each tube. Cap tightly.
4. Autoclave 121°C, 30 min.
5. Allow samples to cool to room temperature.
6. Add 3.3 ml 1 M NaOH to each sample.
7. Cap tubes tightly, and invert to mix the floc produced by the NaOH addition.
8. Centrifuge at 1000g at room temperature for 60 min.
9. Aspirate the supernatant using a clean Pasteur pipette attached to a vacuum line and collection flask. Take care not to disturb the pellet.
10. Add 9.0 ml of 0.1 M HCl to each tube and vortex to dissolve the pellet completely.

11. Add 1 ml reduction mix to each 10 ml sample. Vortex and allow to react for 15±1 min.
12. After 15±1 min add 1 ml molybdenum blue reaction mix to each 10 ml sample and vortex to mix. Allow to react for at least 15 min at room temperature to ensure full color development. Samples and standards are read at 880 nm in 10 cm cells.

Note: Standards are prepared in surface seawater and treated the same as samples. Blank samples are prepared from artificial seawater (Instant Ocean) filtered through 0.2 µm membrane filter. After a MAGIC treatment (2.5% v/v 1 M NaOH addition, centrifuge at 1000g for 1 h), 40 ml aliquots of the supernatant are transferred to glass tubes and treated as regular samples from step 3, above.

◆◆◆◆◆◆ PARTICULATE PHOSPHORUS

Rationale

We define 'particles,' operationally, as those materials that are retained by a Whatman micro-fine glass fiber filter, catalog #GF/F 0.7 µm nominal retention (Sheldon, 1972), acknowledging the fact that some particulate material is known to pass through this filter. The particle retention characteristics are also known to vary with filter loading and, hence, may not be constant even during the filtration of a homogeneous water sample.

In presenting our methods, we have focused on total elemental mass and applications to mass fluxes without consideration as to the chemical form of the PP. Consequently, we derive estimates that cannot be assumed to be equivalent to the organically-bound fraction as they may include inorganic PP as well. More specialized methods do exist for the measurements of lipid-P and nucleic acid-P in the isolated particulate matter (Table 13.1), but these will not be discussed.

The procedure presented herein is a modification of one used by the Hawaii Ocean Time-series (HOT) program at the University of Hawaii and relies on the release of organically-bound phosphorus compounds as *Pi*, by high-temperature combustion at 450°C. The *Pi* is then extracted with HCl at 60°C and neutralized with NaOH. The liberated *Pi* is then measured using the Murphy–Riley procedure described above (see *Pi* determination). This procedure measures all forms of reactive phosphorus released by combustion and acid hydrolysis. Application of this method for PP inventory estimation and for PP fluxes using particle interceptor traps has been presented elsewhere (Karl *et al.*, 1991, 1996).

Particulate phosphorus determination

Equipment and reagents

- Acid-cleaned polyethylene (PE) bottles (12 l)
- Low pressure (4–7 psi nitrogen gas) or vacuum filtration apparatus
- Filter holder assembly for in-line pressure filtration
- Muffle furnace (450°C)
- Drying oven (60°C)
- Combusted (450°C, 4.5 h), HCl (1 M) washed, glass tubes 12 × 75 mm
- Combusted, HCl (1 M) rinsed GF/F filters, 25 mm
- Combusted aluminum foil
- Clean forceps
- Automatic pipettes or repipetter for reagent dispensing
- Hydrochloric acid 0.15 N
- Certified reference material, phosphate standard solutions, molybdenum blue reaction mixture and equipment (all as above for *Pi* determination)

Assay

1. Seawater is collected using appropriate sampling techniques. If necessary, samples are pre-screened through a 202 µm Nitex screen to remove large zooplankton.
2. Samples are filtered through a combusted, acid-rinsed GF/F filter. This can be done by pressure filtration using in-line filter holders and applying 4–7 psi gas pressure (N_2) or by vacuum filtration.
3. Following filtration the filter is transferred into acid-cleaned, combusted glass tubes using clean forceps. Combusted, acid-washed GF/F filters serve as blank samples.
4. The glass tubes are covered with a piece of combusted foil. The foil is secured with label tape and stored frozen (–20°C).
5. Combust samples in muffle furnace (450°C, 4.5 h; remove tape before combusting).
6. After cooling to room temperature, add 10 ml 0.15 N HCl; heat to 60°C for 1 h.
7. Adjust volume to 10 ml, and centrifuge at 1000g for 15 min.
8. Transfer 5 ml of supernatant into acid-washed, combusted glass tube.
9. Add 0.5 ml of the molybdenum blue reaction mixture to each sample; mix and allow to react for 1 h.

Note: Standards are prepared in 0.15 N HCl and treated the same as samples. Samples and standards are read at 880 nm in 1 cm cells. Filter blanks are also included.

Measurements of particulate and dissolved ATP

Rationale: Particulate ATP as a microbial biomass indicator

The phylogenic diversity of microorganisms in the sea and the generally high percentage of non-living to living C, N and P complicates a straightforward assessment of total microbial biomass despite the fundamental importance of this parameter for many ecological studies. Measurements of PP (see above) can be used to provide an upper bound on biomass-P, but in most open ocean surface waters and in all subeuphotic zone waters of the global ocean non-living components dominate the standing stock of POM. It would, therefore, be desirable to have a reliable biochemical or molecular biomarker for life, one that is: (1) present in all living cells and readily metabolized, hydrolyzed or otherwise decomposed following cell death, (2) present in a fixed, constant percentage relative to total cell mass regardless of environmental or physiological conditions; and (3) easily extracted and purified (if necessary) from seawater and conveniently measured.

Levin *et al.* (1964) first suggested that ATP, the common energy currency of all living cells, might be a sensitive indicator for the presence of living microorganisms in natural samples. Two years later, Holm-Hansen and Booth (1966) proposed that ATP measurements could be used to estimate total microbial biomass in marine ecosystems. These pioneering efforts evolved into a simple and extremely sensitive assay that has widescale use in marine microbiology. Without exception, all living organisms contain ATP, so the basis for the use of this molecular biomarker is well-founded (Karl and Dobbs, 1998). The measurement of P-ATP is described in detail elsewhere (Karl, 1993).

Rationale: Dissolved ATP as a 'model' DOP compound

Significant concentrations of D-ATP have been detected in marine (Azam and Hodson, 1977; Nawrocki and Karl, 1989) and freshwater (Riemann, 1979; Maki *et al.*, 1983) environments. It is unknown whether this D-ATP interferes with the conventional measurement of P-ATP in aquatic environments, but since most water samples are generally concentrated onto filters before extraction, the relative contribution from D-ATP should be minimal. The much more interesting questions with regard to the ubiquity of D-ATP in natural aquatic ecosystems are: Why? And by what mechanisms?

Given the fundamental role of ATP in cellular energetics, it is unlikely that D-ATP is derived directly from healthy cells by excretion or exudation. D-ATP production is probably a manifestation of microbial cell death by grazing or autolysis. D-ATP is also a readily bioavailable substrate; marine microorganisms can and do assimilate D-ATP, in part, for P and, in part, for purine salvage and nucleic acid synthesis. Consequently, D-ATP concentration measurements coupled with D-AT^{32}P/^{33}P (or ^{3}H/^{14}C radiolabeled substrates) can be used to estimate D-ATP pool turnover and mass flux through marine microbial assemblages (Azam and Hodson, 1977).

D-ATP is probably the only organic-P compound that can be measured at ambient pool concentrations and can be tracked through the microbial food web maze. In this regard, and given its fundamental role in cellular bioenergetics and biosynthesis, ATP might be considered a model compound in studies of the marine P cycle.

In addition to D-ATP, dissolved ADP and AMP (McGrath and Sullivan, 1981), dissolved cyclic-3′, 5′-AMP (c-AMP; Ammerman and Azam, 1981) and dissolved guanosine-5′-triphosphate (Björkman and Karl, 2000) have also been detected in seawater. At the present time, it is not known whether these related intracellular compounds have similar sources and sinks in the marine environment.

Dissolved ATP

Equipment and reagents

- Acid-cleaned high-density polyethylene (HDPE) sampling bottles
- Vacuum filtration manifold
- Membrane filters (0.2 µm, Poretics, Nuclepore)
- Adjustable automatic pipettes (10–100 µl, 100–1000 µl, 1–5 ml)
- Sodium hydroxide (1 M NaOH, Fluka Biochemika #71689)
- Hydrochloric acid (5 M HCl, Fisher #A144S-212)
- Modified firefly lantern extract (FLE) mixture for high-sensitivity bioluminescence assay:
  1. Firefly lantern extract, freeze dried (Sigma Chemical Co, # FLE-50)
  2. Tris 20 mM, pH 7.4 (Sigma Chemical Co, #T4003)
  3. Arsenate buffer 0.1 M, pH 7.4 (Na_2HAsO_4, Mallinckrodt #7384)
  4. Dithiothreitol 0.1 M (DTT; Sigma Chemical Co, #D-9779)
  5. Luciferin 3.3 mM (Sigma Chemical Co, #L-6882)

  The FLE is reconstituted in 5 ml DDW per 50 mg vial and allowed to age at room temperature for 6–12 h prior to diluting with equal volumes of solutions 2 and 3 to a final volume 75 ml. The FLE mixture is filtered through a GF/F filter to remove solids. Add 1% v/v DTT and luciferin, respectively, to the FLE mixture.
- ATP photometer or equivalent light detection instrument, preferably equipped with an automatic injection unit and interfaced with a computer for calculations of peak height and the integrated counts of the light emission decay curve.

Assay

1. Seawater samples are filtered through 0.2 µm polycarbonate membrane filters and 450 ml of the filtrate transferred to HDPE bottles and stored frozen (–20°C) until further processed. The volume used is determined by the expected dissolved ATP concentration; smaller sample volumes can successfully be used with waters from eutrophic regions.

2. Thaw samples to room temperature in water bath. Transfer to clean transparent bottles.
3. Add 2.25 ml 1 M NaOH (0.5% v/v) to 450 ml sample. Mix thoroughly.
4. Centrifuge at 1000g for 60 min at room temperature.
5. Aspirate supernatant and discard. (If a centrifuge that can accommodate large volume samples is not available the floc can be settled passively for 3 h. Aspirate the overlying volume until approximately 50 ml remains. Transfer this suspended floc to clean 50 ml polypropylene centrifuge tubes and centrifuge as in step #4.)
6. Dissolve pellet by adding 5 M HCl until solution just turns from milky to clear (pH 7–8), usually 100–200 µl. Adjust volume with Tris buffer (20 mM, pH 7.4) to 2 ml.
7. Place 100 µl sample in reaction vial and insert in photometer. Add 800 µl of the FLE reaction mixture and monitor the light emission.

Note: Standards are prepared in deep-sea water with low D-ATP content from stock solutions and treated the same as unknown samples. The blank value is deducted and ATP concentrations are determined from the slope of the standard curve using peak height bioluminescence counts.

◆◆◆◆◆◆ *Pi* UPTAKE/REGENERATION AND DOP PRODUCTION/UTILIZATION RATES

Rationale

The use of stable and radioisotopic tracers to monitor and quantify the rates of microbial growth, metabolism and biogeochemical cycling of key elements and compounds has revolutionized the field of microbiological oceanography. In general, there are two major categories of isotope-based research: (1) the use of naturally occurring stable or radioactive isotopes in selected inorganic substrate pools or organic matter and (2) the use of exogenously supplied stable or radioactive isotopes. The former application, which includes the detection of the cosmogenic radiotracers ^{32}P and ^{33}P (Lal and Lee, 1988; Lal *et al.*, 1988) are most useful for long-term (day to week) whole ecosystem studies. Two recent applications in the Sargasso Sea and Gulf of Maine have demonstrated the efficacy of using natural cosmogenic ^{32}P/^{33}P radioisotopes in studies of the marine P cycle (Waser *et al.*, 1996; Benitez-Nelson and Buesseler, 1999). The latter application, also including the use of exogenously supplied ^{32}P and ^{33}P-labeled compounds, is best suited for short-term (hours to day) studies of metabolic pathways, nutrient fluxes and organic tissue labeling patterns.

The use of ^{32}P (or ^{33}P) as a tracer for P*i* uptake, incorporation and regeneration has been used extensively in oceanography to measure the growth and metabolic activities of algal and bacterial assemblages (Rigler, 1956; Watt and Hayes, 1963; Sorokin and Vyshkvartsev, 1974; Taft *et al.*, 1975;

Harrison *et al.*, 1977; Perry and Eppley, 1981; Sorokin, 1985; Atkinson, 1987; see Table 13.1). Certain P radiotracer field experiments have employed either size fractionation treatments (Harrison *et al.*, 1977), metabolic inhibitors (Krempin *et al.*, 1981) or multiple labeled substrates (Cuhel *et al.*, 1983) to separate algal and heterotrophic bacterial activities. Typically, the radiotracer is added as carrier-free ^{32}Pi (or ^{33}Pi) in order to minimize perturbations that may be caused by P*i* pool enrichment. If accurate determination of ^{31}Pi concentration, or preferably $BA^{31}P$, is obtained then ^{32}Pi (^{33}Pi) uptake rates can be extrapolated to mass fluxes. The key to providing a reliable mass flux is the accurate estimation of the BAP pool. If standard SRP measurements are used, P uptake rates and, hence, mass fluxes could be grossly overestimated or underestimated.

The two radioactive isotopes of P, ^{32}P and ^{33}P, have very different characteristics including half-life and radioactive decay properties. While both isotopes produce β^- particles, the energy of ^{32}P decay is much greater than ^{33}P and the half-life is shorter (^{32}P, E_{max} = 1.710 MeV, half-life = 14.3 days; ^{33}P, E_{max} = 0.249 MeV, half-life = 25.3 days). A consequence of the ^{32}P high energy β^- emission is a phenomenon known as Cerenkov radiation. This property provides a unique opportunity to detect and quantify the activity of ^{32}P in dissolved and particulate matter with reasonable counting efficiencies (~40%) in the absence of a scintillation fluor and, therefore, without chemical quench. This non-destructive counting method means that the entire sample can be recovered for subsequent analysis.

In addition to P*i* uptake and rates of P incorporation into PP or specific subcellular pools (e.g. lipids, nucleic acids; see Table 13.1), exogenous radiotracer experiments can also provide information on P*i* regeneration rates (Harrison, 1983; Smith *et al.*, 1985) and DOP production rates (Johannes, 1964; Lemasson and Pages, 1981; Orrett and Karl, 1987; Björkman *et al.*, 2000). As for P*i* uptake, accurate P*i* regeneration or DOP production estimation requires information on the ambient pool of BAP in the habitat under investigation. When combined with independent methods of DOP pool characterization, these radiotracer experiments can also be used to quantify the fluxes of specific DOP compounds or compound classes (e.g. nucleotides). A 'pulse-chase' experimental design (i.e. pre-incubation with ^{32}Pi or ^{33}Pi radiotracer followed by the addition of a ten-fold excess of ^{31}Pi) could be employed to follow both net DOP production, during the labeling phase, and $DO^{32}P$ turnover in the post-chase treatments. Finally, the addition of exogenous, ^{32}P (or ^{33}P)-radiolabeled DOP compounds (e.g. glucose-P, ATP, glycerol-P) can provide information on the turnover time of individual pools and potentially (if combined with direct measurements of ambient pool concentrations) on mass fluxes (production and utilization rates) through selected organic-P pools. Our present inability to measure any but a very few DOP compounds (a notable exception is ATP, as discussed above) limits the application of this experimental design.

Pi uptake and DOP production

Equipment and reagents

- Acid-cleaned high density polyethylene (HDPE) sampling bottles
- Polypropylene centrifuge tubes 50 ml (Corning)
- Polycarbonate incubation bottles
- On-deck temperature- and light-controlled incubator, or *in situ* incubation array
- Vacuum filtration manifold, filtration funnels and bases, vacuum pump
- Membrane filters (0.2 µm Poretics, Nuclepore)
- Glass scintillation vials
- Acid cleaned autoclavable glass centrifuge tubes 50 ml with teflon lined caps (Kimble)
- Plastic pipettes (50 ml) for sample dispensing
- Automatic pipettes or repipetter for reagent dispensing and sampling
- Centrifuge with capacity to hold 50 ml tubes
- Steam or electric autoclave
- Radiolabeled orthophosphate ($^{32}Pi/^{33}Pi$) as a stock solution of approximately 18.5 MBq ml^{-1} (500 µCi ml^{-1}) (ICN Radiochemicals)
- Sodium hydroxide 1 M (NaOH, Fluka Biochemica #71689)
- Potassium persulfate 5% w/v ($K_2S_2O_8$, Fisher #P281)
- Hydrochloric acid 0.1 M (Fisher trace metal grade HCl, #A508-212)
- Spectrophotometer Beckman DU 640, or equivalent, and cuvette cells (10 cm)
- Liquid scintillation counter (LSC)
- Arsenate reduction mixture, *Pi* standards, molybdenum blue reaction mixture and equipment (as above for *Pi* determination)

Assay

1. Incubate freshly collected seawater samples under defined temperature and light conditions in the presence of the radiolabel. The radioactivity required depends on sample size and ambient BAP concentrations.
2. At predetermined incubation periods, subsamples are removed for total (1 ml whole water) and particulate radioactivity by filtration onto membrane filters. The whole water and filter samples are placed in scintillation vials and the filtrate collected and stored frozen (–20°C) in HDPE bottles.
3. The filters and whole water samples are counted by LSC.
4. The filtrate samples are thawed in a water bath to room temperature.
5. Triplicate 50 ml sample aliquots are placed into new 50 ml polypropylene centrifuge tubes using a 50 ml plastic pipette; 1 ml from each sample is placed directly into a scintillation vial for LSC counting of total ^{32}P (^{33}P) activity.
6. Add 150 µl (0.3% v/v) 1 M NaOH to each 50 ml aliquot.

7. Cap tubes tightly, and invert to mix the floc produced by the NaOH addition.
8. Centrifuge at 1000g at room temperature for 60 min.
9. Remove 1 ml of supernatant for LSC counting of remaining radioactivity ($DO^{32}P/DO^{33}P$).
10. Transfer 3 × 40 ml of the supernatant to 50 ml acid washed glass centrifuge tubes using a 50 ml plastic pipette.
11. Aspirate the remaining supernatant in the polypropylene tubes taking care not to disturb the small pellet (go to step # 20).
12. To each glass tube add 6.4 ml potassium persulfate solution. Cap tightly.
13. Autoclave 121°C, 30 min.
14. Allow samples to cool to room temperature.
15. Add 3.3 ml 1 M NaOH to each sample.
16. Cap tubes tightly, and invert to mix the floc produced by the NaOH addition.
17. Centrifuge at 1000g at room temperature for 60 min.
18. Aspirate the supernatant using a clean Pasteur pipette attached to a vacuum line and a collection flask.
19. Add 9.0 ml of 0.1 M HCl is added to each tube and shake to dissolve the pellet.
20. Add 1 ml reduction mix to each 10 ml sample. Measure P*i*, as above.

Note: From time course incubations the uptake rate of $^{32}P_i$, and the turnover time of the P*i* equivalent pool can be calculated. If the P*i* (SRP or BAP pool) is known the mass flux of P can be calculated from the turnover time and the known concentration of the relevant P-pool. The production of DOP is estimated from the increase in ^{32}P activity in the supernatant over time and by assuming that the DOP formed has the same specific activity as the initial P*i*. The production rate of DOP can be calculated and the average turnover time for the DOP pool assessed with the knowledge of the DOP pool size derived from the DOP concentrations in the samples.

◆◆◆◆◆◆ INTRACELLULAR ATP POOL TURNOVER AND BIOLOGICALLY AVAILABLE P

Principle: Intracellular ATP pool turnover

The central role of ATP in the stoichiometric coupling of energy-yielding and energy-requiring metabolic reactions has been known since the pioneering research of Lipmann (1941). The steady-state intracellular concentration of ATP in viable microorganisms (prokaryotes and eukaryotes) appears to be well-regulated at a value of 2 to 6 nmol ATP mg^{-1} dry weight (~1 to 3 mM) regardless of growth rate, culture condition, or mode of nutrition (Karl, 1980). Previous studies of ATP pools in microorganisms

and in environmental samples have emphasized the futility of extrapolating these static measurements to estimates of metabolic energy flux. Consequently, it is the turnover rate of the ATP pool, rather than the steady-state concentration of that pool, which varies in proportion to cellular metabolic energy requirements. It follows then, that direct measurements of ATP pool turnover rates, when coupled with independent estimates of ATP pool size, should provide useful information on biological energy flux in cells, populations, or natural microbial assemblages.

Cellular ATP pool turnover results from the hydrolysis of one or both of the anhydride-bound PO_4 groups (the β-P and γ-P or 'high-energy' phosphate bonds) via orthophosphate or pyrophosphate cleavage, followed by subsequent regeneration of ATP by either substrate-level, oxidative, or photophosphorylation processes. Because ATP pool turnover defines a cycle, this essential set of metabolic processes results neither in net removal of adenine nucleotide molecules from the intracellular pool nor requires coupled biosynthesis thereof. This is important in the analytical procedures employed to distinguish between the turnover cycles of ATP and total adenine nucleotide (TAN) pools (Karl, 1993).

In theory, ATP pool turnover rates could be measured under steady-state metabolic conditions (i.e. $d[ATP]/dt = 0$) by estimating either the rate of ATP formation or rate of ATP utilization, if either process could be temporarily suspended without affecting the other. Such ATP pool transitions have been observed in microorganisms following the rapid removal of oxygen (for aerobic heterotrophs) or light (for phototrophs), but it is uncertain whether the results derived from these harsh experimental perturbations yield reliable estimates of ATP pool dynamics. Karl and Bossard (1985a) devised a novel method for ATP pool turnover in cell cultures or natural populations of microorganisms. The procedure relies upon the use of $^{32}P_i$ (or $^{33}P_i$) as a tracer for P-flux through the acid anhydride-bound P (β-P and γ-P) groups of cellular ATP. The uptake of $^{32}P_i$ also results in the labeling of the α-P group of ATP, if the population in question is actively growing (i.e. if there is a net removal of adenine nucleotides for biosynthesis). Consequently, in order to uniquely assess the labeling of ATP derived from energy flux, one must be able to separate β-P and γ-P labeling from α-P (Karl and Bossard, 1985a). On the other hand, α-P labeling defines TAN pool turnover and therefore, $^{32}P_i$ labeling can be used to estimate both ATP and TAN pool turnover rates.

The uptake, or salvage, of exogenously added nucleic acid precursors (e.g. adenine, thymidine, uridine) in preference to *de novo* synthesis is a well-documented characteristic of aquatic microbial communities (Karl, 1979; Fuhrman and Azam, 1980). This phenomenon comprises the theoretical basis for the use of [^{3}H]adenine, [^{3}H]thymidine, and [^{3}H]uridine in ecological studies of nucleic acid synthesis. An important aspect of the incorporation of nucleic acid precursors is the observation that the total flux of precursor into the nucleotide triphosphate pools (i.e. ATP, TTP, UTP, etc.), which is the result of the combined effects of salvage and *de novo* synthesis, is in equilibrium with the removal of the triphosphate precursors required for cellular biosynthesis. Furthermore, it is well

documented that the TAN pool (i.e. steady-state intracellular concentration of [ATP + ADP + AMP]) in microorganisms does not vary with changes in the specific growth rate (Karl, 1980; Chapman and Atkinson, 1977). Because cellular biosynthesis is directly related to growth rate, a positive correlation is expected to exist between cellular adenine nucleotide flux and growth. It follows then that the turnover rate of the TAN pool (and most likely, of all ribonucleotide and deoxyribonucleotide pools in general) must vary in direct proportion to the rates of nucleic acid synthesis and hence, net growth.

The TAN pool turnover time is defined as the average residence time of a molecule in the intracellular pool before it is removed for macromolecular biosynthesis (i.e. the steady-state pool concentration divided by the steady-state rate of synthesis or removal). If a radioactive precursor such as [^{3}H]adenine is added to a growing culture or natural population of microorganisms, the TAN pool turnover time can be calculated by monitoring the change in the specific activity (SA) of the ATP pool with incubation time. TAN pool turnover rate can also be measured by monitoring the labeling kinetics of the α-P position of ATP following the addition of ^{32}Pi (or ^{33}Pi) to a sample (Karl and Bossard, 1985b). Exactly one turnover cycle has been completed when the ATP pool has achieved an SA that is equal to 50% of the value at isotopic equilibrium. Chapman and Atkinson (1977) have estimated TAN pool turnover rates as a function of growth rate for *Escherichia coli* and *Salmonella typhimurium*. They conclude that the TAN pool 'turns over' (i.e. is completely utilized for biosynthesis and is replenished through salvage and *de novo* synthesis) 30 to 50 times per generation regardless of generation time. This prediction has been tested using a diverse variety of marine microorganisms (Karl *et al.*, 1987). The results indicate that the TAN pool turnover time is positively correlated with generation time and averages 2.2% of the generation time (i.e. TAN pool turns over 45 times per generation).

Consequently, a single time-course incubation with ^{32}Pi (^{33}Pi), followed by isolation and selective hydrolysis of the ATP pool, can be used to assess the independent labeling kinetics of the α-P, β-P and γ-P moieties of ATP. The turnover rate of the α-P position provides quantitative information on TAN pool turnover and, hence, growth rate, and the turnover rate of the β-P and γ-P positions provides quantitative information on ATP pool turnover and, hence, energy flux. The labeling of γ-P can also be used to estimate the BAP pool (see following section). Specific ecological applications of the ATP pool and TAN pool turnover for microbial growth rate and energy flux determinations have been presented elsewhere (Karl and Bossard, 1985a,b; Bossard and Karl, 1986; Laws *et al.*, 1986; Karl *et al.*, 1987; Karl, 1993).

Principle: Biologically available P

Regardless of the rigor and precision with which P-containing pools are measured, the ecological significance of these analytical determinations will be incomplete until reliable estimates of the BAP pool are routinely

available. In addition to P*i*, which is generally the preferred substrate for microorganisms, the P contained in a variety of polymeric inorganic compounds, in monomeric and polymeric organic compounds and in selected P-containing minerals is available to some or all microorganisms; indeed some microorganisms may prefer ester-linked P sources to free orthophosphate (Tarapchak and Moll, 1990; Cotner and Wetzel, 1992). However, the bioavailability of most organic-P pools depends critically on ambient P*i* pool concentrations and on the expression of specific transport, salvage and hydrolytic enzymes. Because many of these enzymes are induced by low P*i*, bioavailability may be a variable, time- and habitat condition-dependent parameter, rather than an easily predicted or measured metric. Assessment of the BAP may also depend on the timescale of consideration: substrates that appear to be recalcitrant on short timescales (e.g. < 1 day) may readily fuel longer-term (annual to decadal) microbial metabolism.

BAP can be estimated using the γ-P labeling technique described later for ATP pool turnover. Because the intracellular ATP pool turns over rapidly, the P-specific radioactivity in the γ-P position of the P-ATP pool will reach equilibrium not only with the extracellular P*i* pool but with the potentially larger pool of extracellular P that is available to the microbial assemblage at that time, the so-called BAP pool. The only difference between the determination of ATP γ-P labeling and estimation of BAP is that the latter determination demands a direct measurement of the total P-ATP pool, as well as the amount of ^{32}P (or ^{33}P) contained in the isolated γ-P fraction thereof. This provides the data necessary to calculate the P-specific radioactivity of the γ-P in the intracellular ATP pool (i.e. ^{32}P (or ^{33}P) mol^{-1}) which, at isotopic equilibrium (i.e. five turnover cycles) is identical to the specific radioactivity of the extracellular BAP pool.

ATP pool turnover and BAP

Equipment and reagents

- Acid-cleaned polycarbonate incubation bottles (0.1 to 4 l, depending on experimental design)
- On-deck temperature- and light-controlled incubator, or *in situ* incubation array
- Filtration gear, heating block and other ATP extraction and detection equipment and supplies
- Vacuum evaporator (Savant Speed-Vac or equivalent)
- Microcentrifuge and gyrotatory shaker table
- Thin layer chromatography (TLC) glass tanks with covers
- TLC plates (20 × 20 cm), polyethyleneimine (PEI) impregnated cellulose (Polygram CEL 300 PEI, Machery-Nagel, Fisher #NC-9254648)
- High-intensity ultraviolet (UV) lamp
- Radiolabeled orthophosphate ($^{32}PO_4$ or $^{33}PO_4$) as a stock solution of approximately 18.5 MBq ml^{-1} (500 μCi ml^{-1}) (ICN Radiochemicals)

- ATP (0.5 mM in Tris buffer 20 mM, pH 7.4, Sigma #A-6144)
- Formic acid (0.2 M, Baker #1-0128)
- Potassium phosphate solution (0.85 M, pH 3.4, Fisher #P288)
- Magnesium chloride (0.35 M, Fisher #M87)
- Activated charcoal slurry: 25 mg charcoal per ml of 0.1 M H_3PO_4 (activated charcoal, Sigma #C-4386)
- ATPase, porcine cerebral cortex (EC 3.6.1.3; Sigma Chemical Co, #A7510) prepared in distilled deionized water (DDW) to a concentration of 1 unit ml^{-1} and stored frozen at –20°C until needed. Alternatively apyrase (Sigma Chemical Co, #A6132) can be used (10 units m^{-1} in DDW) and stored frozen
- Liquid scintillation counter (LSC)

Assay

1. Incubate freshly collected seawater samples under defined temperature and light conditions in the presence of exogenous ^{32}Pi (or ^{33}Pi); typically 20–40 MBq l^{-1}.
2. At predetermined incubation periods, subsamples are removed and particulate ATP pools are extracted in boiling Tris buffer and stored frozen.
3. Thaw samples; centrifuge at 1000g for 15 min to remove filter debris.
4. Transfer 3.5 to 4.0 ml of the supernatant to clean scintillation vials.
5. Evaporate to dryness *in vacuo* (Speed Vac). The samples are then reconstituted in 100–200 µl DDW.
6. In clean disposable glass tube mix 10 µl of concentrated ATP extract with 10 µl of non-radioactive ATP (0.5 mM) solution. Spot 10 µl of this mixture on predetermined location approximately 1 cm from bottom edge on a PEI plate. About 8–10 lanes can be prepared per plate.
7. Air-dry plate and then wash twice (5 min each) by immersion in DDW (1 l). Allow plate to dry (this step can be accelerated by using a hairdryer).
8. Develop plate in formic acid (0.2 M) to top of plate in a closed TLC chamber.
9. Remove and immediately immerse in DDW (1 l) for 5 min. Allow to dry.
10. Develop plate in the same direction in potassium phosphate solution (0.85 M, pH 3.4) to approximately 2 cm from top edge of plate in closed TLC chamber.
11. Wash plate (2 × 5 min, 1 l DDW) and air dry.
12. The ATP spots are visualized under UV-light, circled with a pencil and cut out with a pair of scissors. Recovery is typically >90%.
13. The excised PEI cuttings are placed into scintillation vials containing 1 ml of 0.35 M $MgCl_2$ and placed on shaker table for 1 h to elute ATP from the PEI matrix.

14. A 10 µl aliquot from the elute is diluted into 1 ml Tris buffer (20 mM, pH 7.4) and assayed for total ATP by the firefly bioluminescence reaction. This is to determine the recovery of the ATP initially applied to the PEI plate.
15. To perform the ATPase hydrolysis reaction place 750 µl of the 'TLC'-purified ATP solution (in 0.35 M $MgCl_2$) into a 12 × 75 mm glass culture tube containing; add 250 µl DDW, 10 µl KCl (1 M), 20 µl NaCl (5 M) and 25 µl AMP (3 mM).
16. Add 50 µl of the ATPase solution (stock at 1 unit ml^{-1}). Mix thoroughly.
17. Immediately remove 10 µl of mixture and dilute into 1 ml Tris (20 mM, pH 7.4) to quench the reaction and assay for 'time zero' ATP (as above for ATP determination).
18. The ATP procedure is repeated (10 µl sample, as above) after 10 to 15 min with one or two representative samples to establish the initial rate of hydrolysis of ATP to ADP. Once the rate is known the time for complete hydrolysis can be calculated (normally 20–40 min). The rate of hydrolysis is expected to be similar, however, complete ATP hydrolysis should be confirmed for each sample before proceeding with the separation and purification of products.
19. Remove 250 µl of the sample and place in scintillation vial for LSC counting of the 'total radioactivity' (i.e. α-, β- and γ-^{32}P of ATP).
20. Place a second 500 µl aliquot into a 1.5 ml microcentrifuge tube containing 500 µl of the charcoal slurry. Mix thoroughly (vortex).
21. Centrifuge at 13 000g for 10 min.
22. Remove 750 µl of supernatant is placed into a scintillation vial for LSC counting of the γ-^{32}P-activity.
23. Calculate the specific radioactivity of the ATP pool over time.
24. The specific γ-^{32}P-activity of the intracellular ATP pool when it has reached its isotopic equilibrium (i.e. maximum) corresponds to the specific activity of the precursor pool (i.e. the bioavailable P (BAP) pool) and the size of the BAP pool can be determined.

Note: If apyrase is used instead of ATPase in step #15; place 750 µl of the 'TLC'-purified ATP solution (in 0.35 M $MgCl_2$) into a 12 × 75 mm glass culture tube containing; 250 µl DDW, 20 µl $CaCl_2$ (1 M). Add 25 µl apyrase (10 units ml^{-1}) and mix thoroughly. Proceed from step 21. The apyrase cleaves both the β- and γ-^{32}P of ATP and this has to be taken into account when calculating the specific activity of the intracellular ATP pool and in estimating the size of the BAP pool.

Calculations: From the data obtained above, the turnover time of the total ATP (β- and γ-^{32}P labeling) and or total adenine (TAN) pool (α-^{32}P labeling) can be calculated and from that energy flux and community growth rate respectively. The change in the ATP or TAN pool-specific activity (SA; nCi $pmol^{-1}$), predicted by radiotracer theory, follows an exponential function of the incubation time. The decimal equivalent of SA at any time (SA_t) can be described by the equation: $SA_t = 1 - (2^{-N})$,

where N is the number of turnover cycles observed during the incubation period. ATP or TAN pool turnover time (T) can be calculated from the expression: $T = t/N$. At incubation times ≥ 5 the pools are in isotopic equilibrium and would, in theory, not change until the exogenous precursor source is exhausted, and pool delabeling begins. Once T has been determined it can be used to extrapolate energy flux (EF) and specific growth rate: EF (kcal l^{-1} h^{-1}) = –22 ([ATP]/T_{ATP}), where [ATP] is equal to the total particulate ATP pool (M) and T_{ATP} is ATP pool turnover time (h). To estimate growth rate, the TAN pool turnover time is assumed to be equivalent to 2–3% of the generation time (i.e. the doubling time (T_d) is on average $45 \times T_{TAN}$).

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