

# Analysing the complexity of marine food webs using amino acid stable nitrogen isotopes: from trophic positions to diets

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# Program

- Objectives: origin, modification and fate of C and N
- Advantages over bulk measurements



- the basis: differential fractionation



- Molecular and isotopic analysis: molecular isolation



- Estimation models: trophic position and other indicators



- Applications: examples from pelagic ecosystems



- Progress: multitrophic models



- Diet/habitat fingerprinting: C and N in amino acids

# Objectives

origin, modification and fate of C and N

## Quantifying the structure and function of food webs

- trophic position ( $TP$ )
- food chain length (=  $TP$  top predator)
- heterotrophic resynthesis ( $\Sigma V$ )
- microbial food web contribution
- diet quality and nitrogen recycling effects

# Definitions

## *Bulk Stable Isotope Analysis = BSIA*

- natural abundance of stable isotopes in bulk tissues / organisms
- minimal sample preparation (drying, grinding)
- elemental analyzer -> mass spectrometer
- lower costs
- popular and accesible

## *Compound-specific amino acid analysis = CSIA-AA, CSIAA*

- natural abundance of stable isotopes in amino acids
- requires prior separation of amino acids (hidrolysis, esterification, derivatization)
- gas (liquid) chromatograph -> mass spectrometer
- higher costs
- available only at few laboratories

# Advantages

## TP estimations using $\delta^{15}\text{N}$

### BSIA

$$TP_c = \lambda + (\delta^{15}\text{N}_c - \delta^{15}\text{N}_b) / TDF$$

TP baseline

baseline

trophic discrimination factor

- requires external baseline ( $\lambda$ )
- variable *trophic discrimination factor* (*TDF*)
- higher uncertainty in *TP*

### CSIAA

$$TP_c = 1 + (\delta^{15}\text{N}_t - \delta^{15}\text{N}_s - \beta_{t/s}) / TDF_{t/s}$$

TP baseline

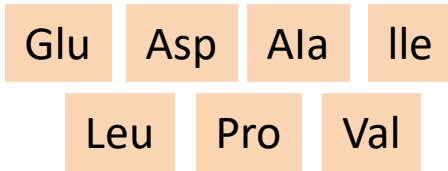
source AA offset

trophic discrimination factor

- internal baseline
- constrained *TDF* (by consumer type)
- constrained  $\beta$  (by primary producer type)
- lower uncertainty in *TP*
- more trophic information ( $\Delta\Sigma V$ , microbial web, diets,...)

# The basis of CSIAA

## trophic AA

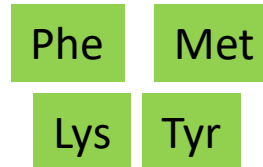


isotopic  
fractionation

high

break / creation of C-N bonds  
(transamination / desamination)

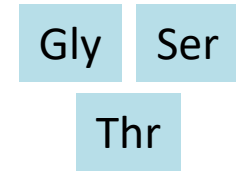
## source AA



low

conservation of C-N  
bonds (hidroxilation)

## 'metabolic' AA

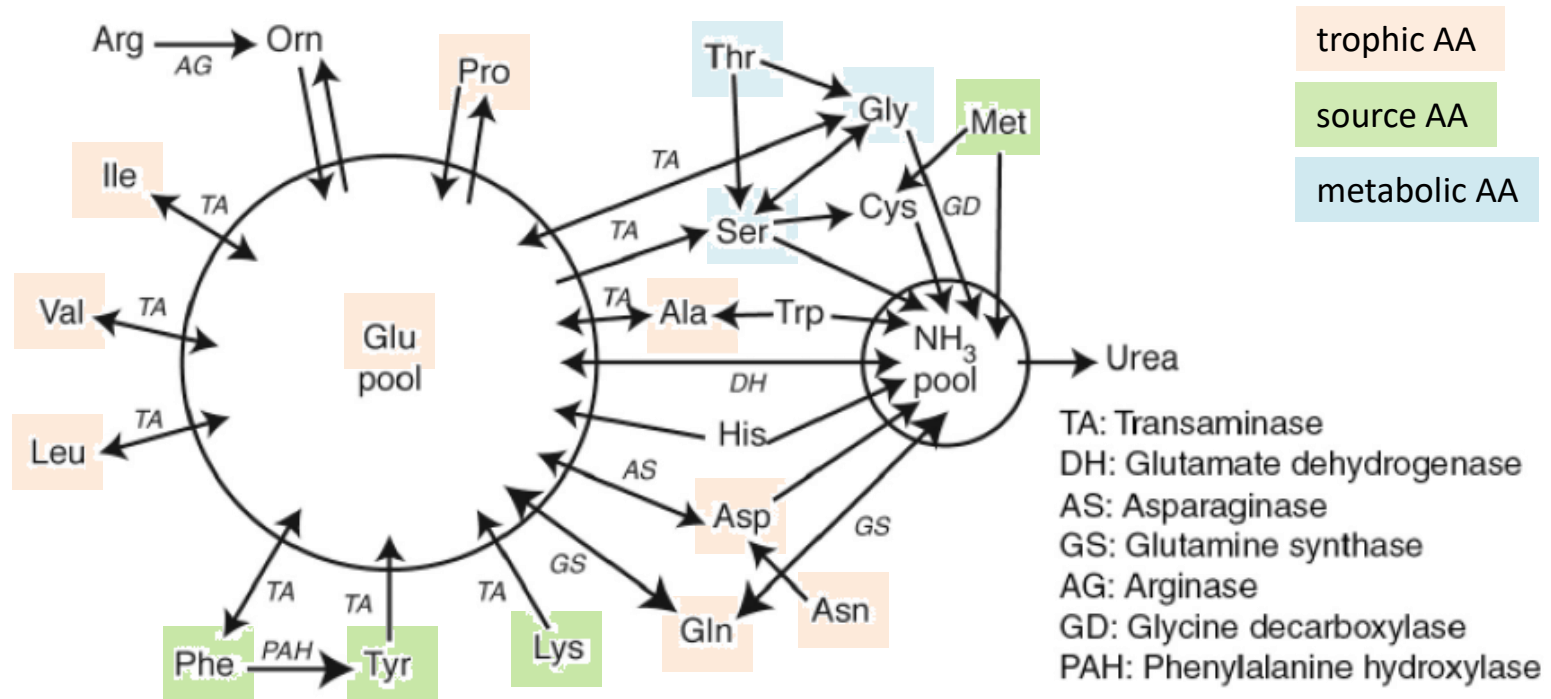


variable

exchange of C-N bonds  
inverse discrimination?

# The basis of CSIAA

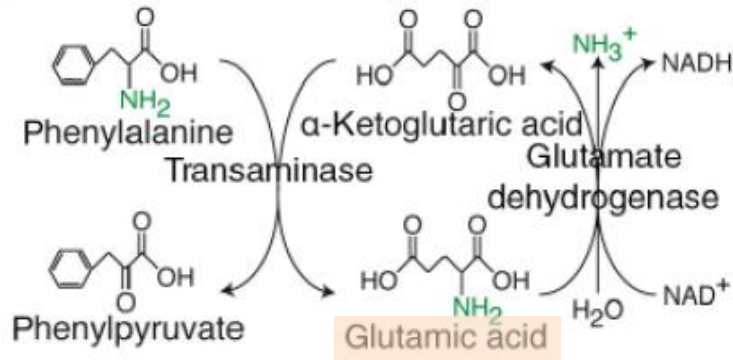
## AA metabolism



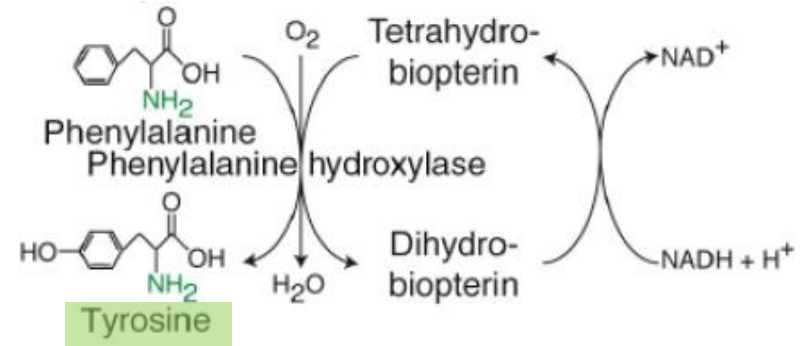
[McMahon & McCarthy, 2016](#)

# The basis of CSIAA

trophic AA: transamination and  
oxidative deamination



source AA: oxidation from Phe to Tyr

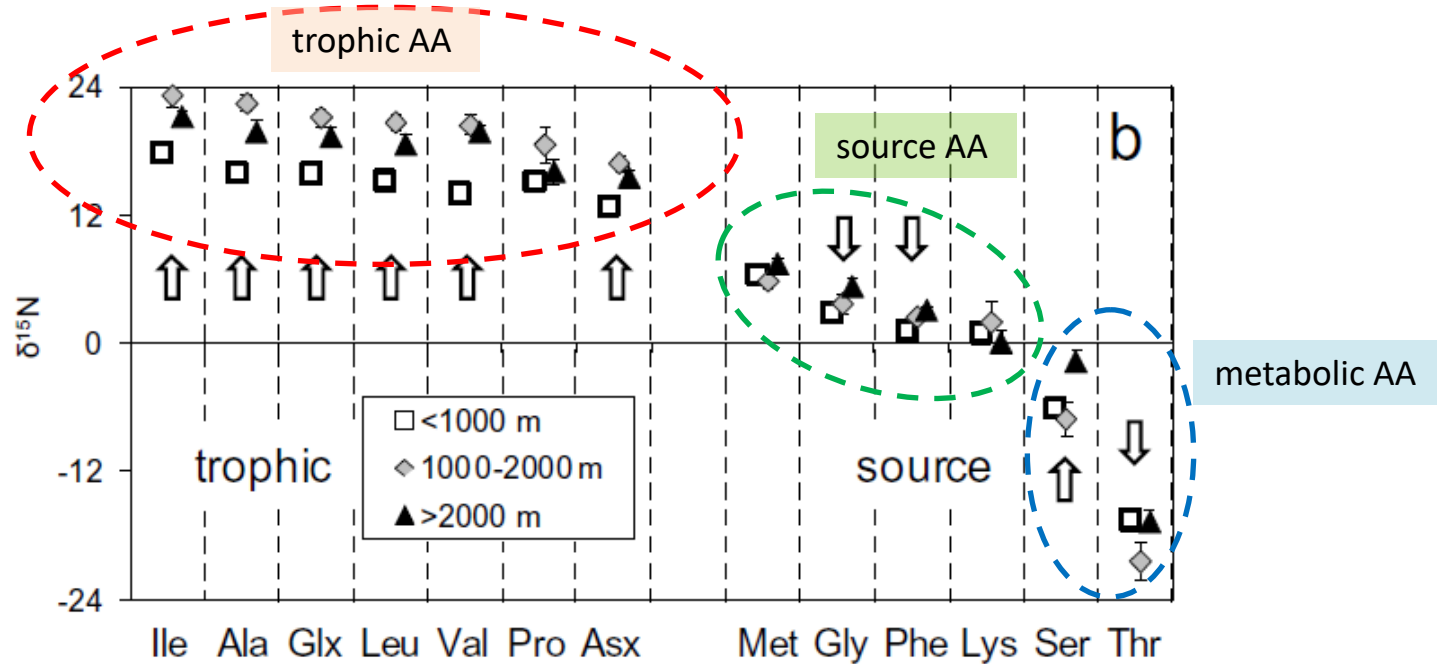


[McMahon & McCarthy, 2016](#)



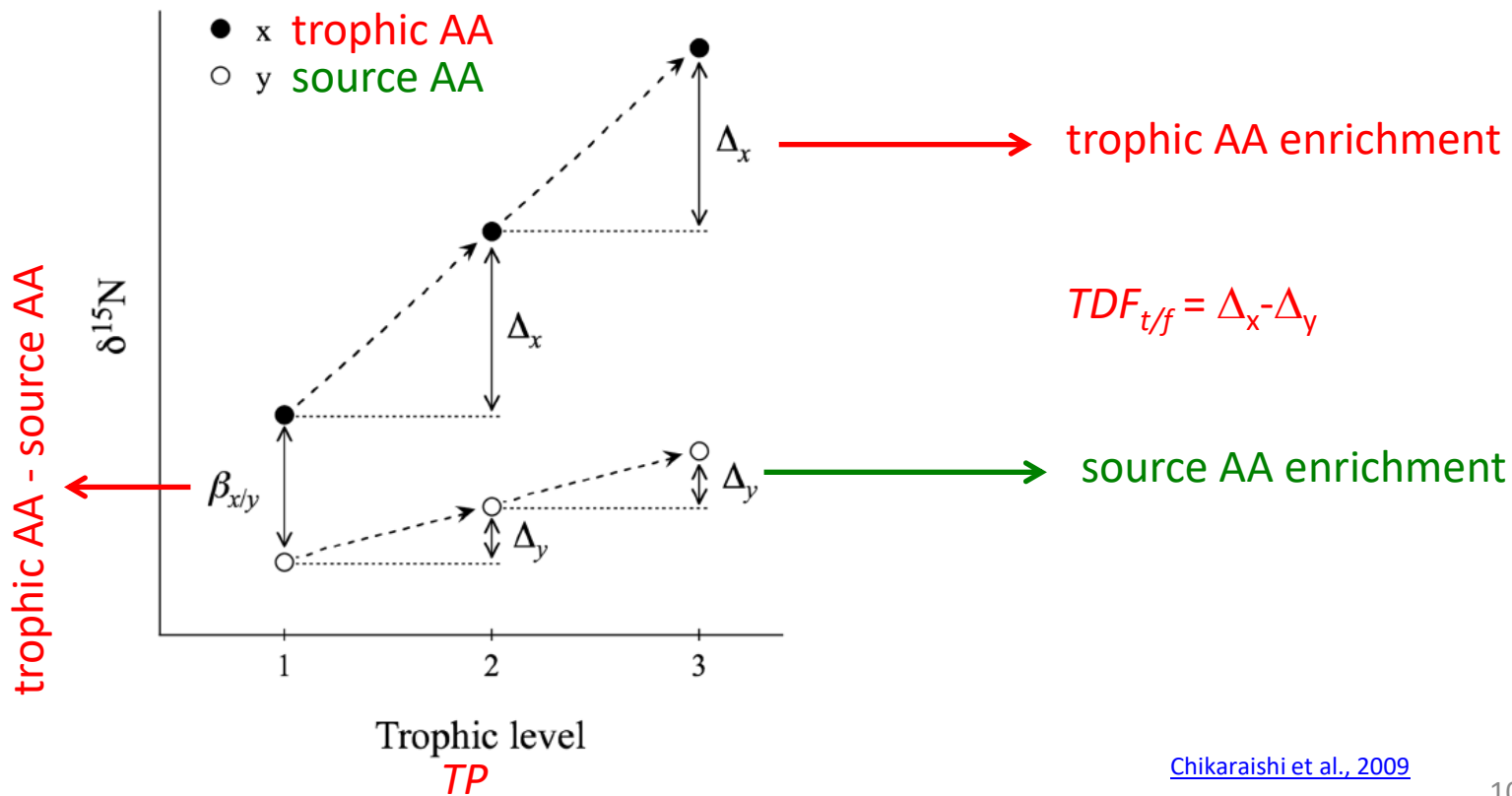
# The basis of CSIAA

example of  $\delta^{15}\text{N}_{\text{AA}}$  in mesopelagic fish



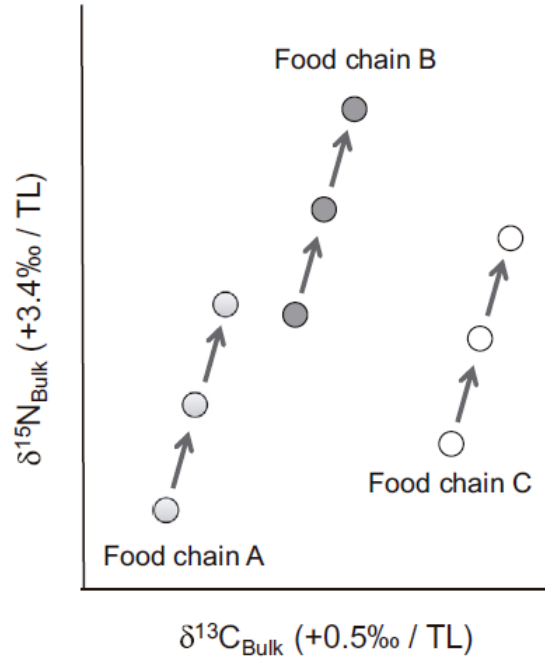
# The basis of CSIAA

$$TP_c = 1 + (\delta^{15}N_t - \delta^{15}N_s - \beta_{t/s}) / TDF_{t/s}$$



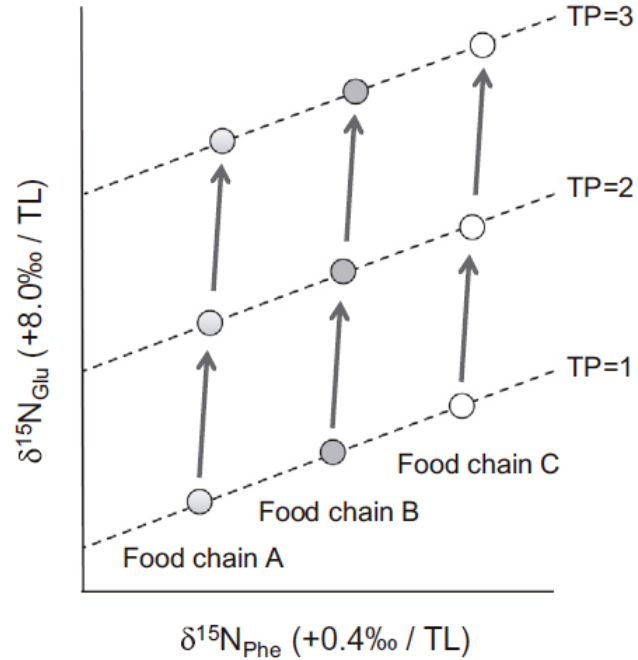
# The basis of CSIAA

(a) Bulk method



depends on N sources

(b) CSIA-AA method



independent from N sources

# The basis of CSIAA

## a short history

- [McClelland & Montoya, 2002](#) ->  $AA_t$  -  $AA_s$  distinction; trophic implications
  - [Popp et al. 2007](#) ->  $AA_t$  -  $AA_s$  definition; application to predator  $TP$
  - [Chikaraishi et al. 2009](#) ->  $TP$  estimation model; definition of universal  $\beta_{t/s}$  and  $TDF_{t/s}$
  - [Nielsen et al. 2015](#) ->  $TDF$  review; averaged  $TDF$
  - [Bradley et al. 2015](#) -> multi- $TDF$  model
  - ➔ [McMahon & McCarthy, 2016](#) -> diet and N excretion mode affect  $TDF$
  - [Decima et al. 2017](#) ->  $TDF_{Ala}$  'visibilizes' microbial trophic steps
  - ➔ [Ohkouchi et al. 2017](#) -> review of  $TDF$  patterns
  - [Ishikawa et al. 2018](#) -> Met as diagnostic for source primary producers
  - [Ramirez et al. 2021](#) -> review of  $\beta_{t/s}$  patterns
  - [García-Seoane et al. \(in review\)](#) -> weighted average of  $\beta_{t/s}$
- terrestrial  
aquatic plants  
algae

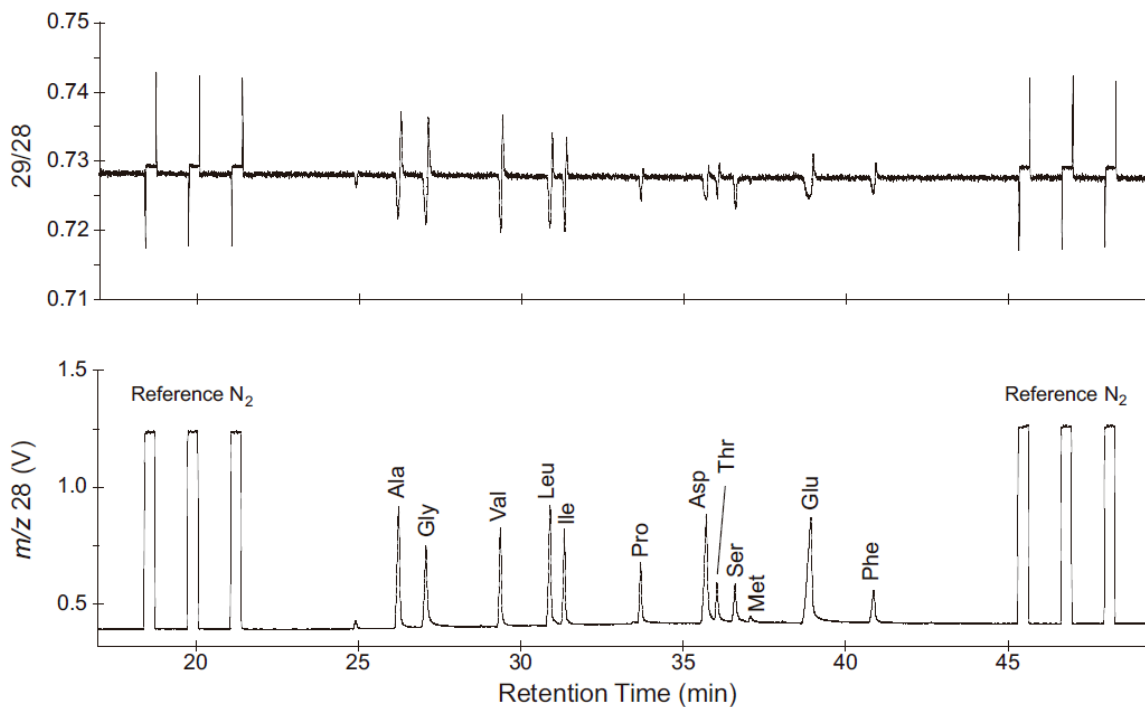
# CSIAA analysis

## gas chromatography (GC)

- sampling tissues (muscle, ...)
- conservation / drying
- preparation:
  - homogenization (grinding)
  - (purification: e.g. removal of lipids, carbonates,...)
  - hidrolisis
  - esterification
  - derivatization
- analysis:
  - gas chromatography (GC)
  - mass espectrometry (Isotope Ratio Mass Spectrometry -IRMS)

# CSIAA analysis

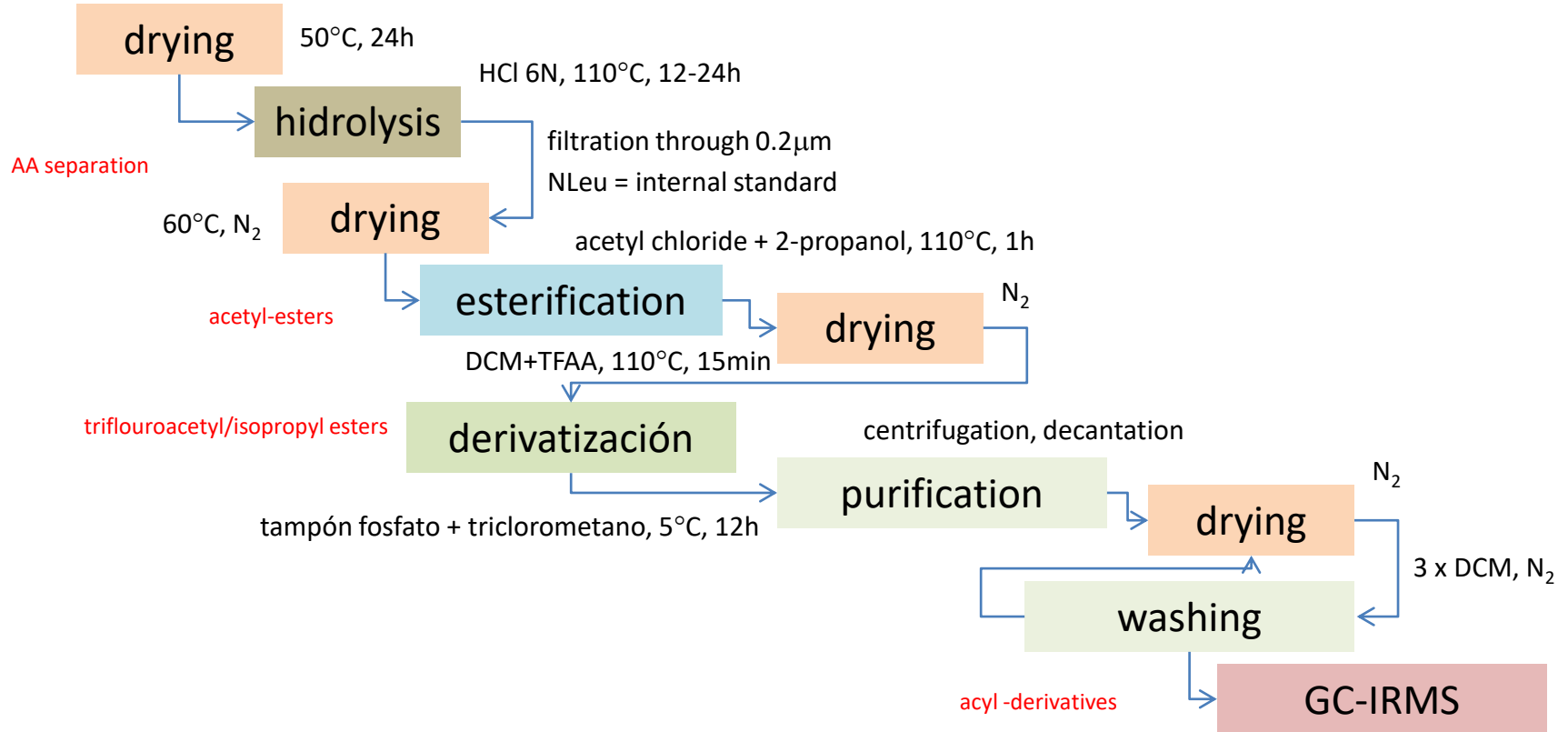
## GC-IRMS chromatograms



# CSIAA analysis

## example of process flow

[McCarthy et al. 2013](#)



[Bode et al. 2021a](#)

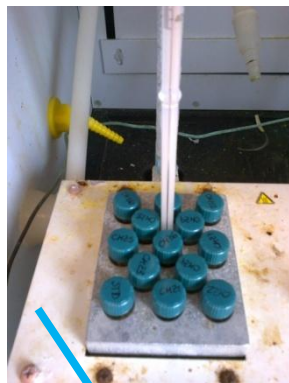
# CSIAA analysis

preparation - hidrolisis - esterificacion - derivatization

hidrolisis



sample preparation and standard addition



esterificacion



evaporation



derivatization



chromatography and IRMS

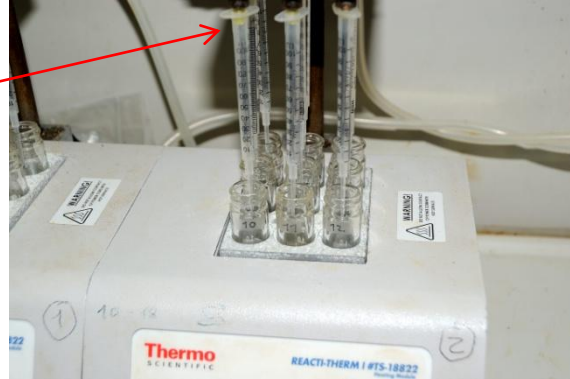
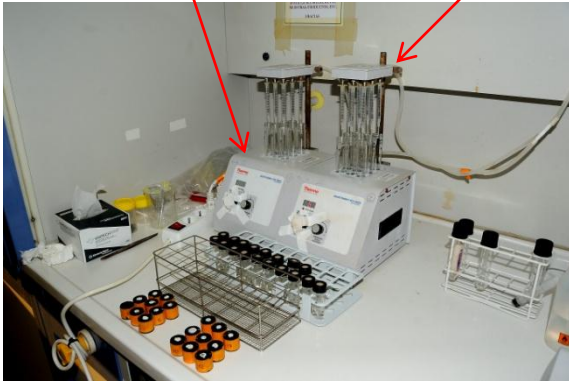




# CSIAA analysis

preparation - hidrolisis - esterificacion - derivatization

heating blocks and evaporators using  $N_2$



$N_2$  difusion tip

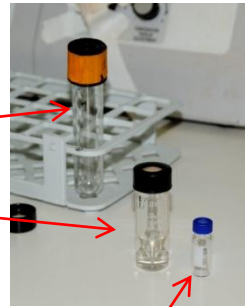


filtration-ash removal



hidrolisis vial

esterificacion-derivatization vial



analysis vial

# Estimation models

trophic position

$$TP_c = 1 + (\delta^{15}N_t - \delta^{15}N_s - \beta_{t/s}) / TDF_{t/s}$$

Diagram illustrating the trophic position estimation model. The equation is enclosed in a blue box. Labels with arrows point to components of the equation: 'baseline TP' points to the '1', 'source AA' points to  $\delta^{15}N_s$ , 'source AA offset' points to  $\beta_{t/s}$ , 'trophic AA' points to  $\delta^{15}N_t$ , and 'trophic discrimination' points to  $TDF_{t/s}$ .

trophic AA (t): Glu (Glx = Glu+Gln)  
source AA (s): Phe

$$\beta_{Glu/Phe} = \delta^{15}N_{Glu} - \delta^{15}N_{Phe} \longrightarrow 3.4\text{‰}$$

in primary producers

in aquatic ecosystems

$$TDF_{Glu/Phe} = \Delta (\delta^{15}N_{Glu} - \delta^{15}N_{Phe})$$

between trophic levels

7.6‰

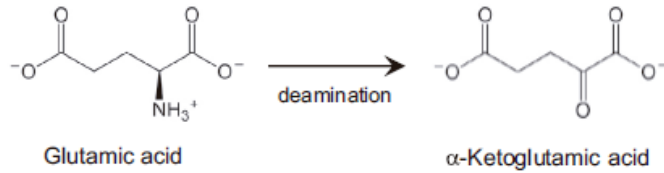
-8.4‰

in terrestrial ecosystems

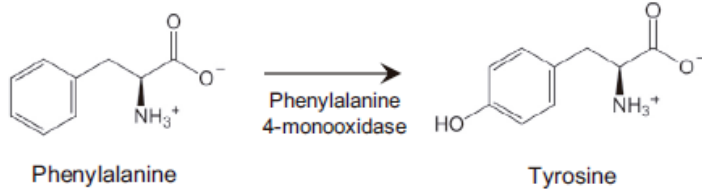
# Estimation models

## trophic position

(a) First step in metabolism

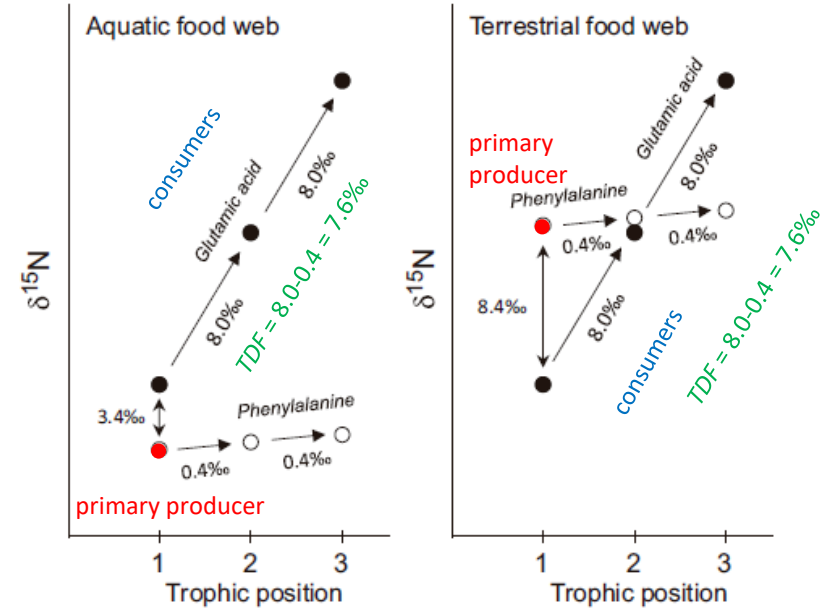


trophic AA



source AA

(b) Trophic enrichment in  $^{15}\text{N}$



# Estimation models

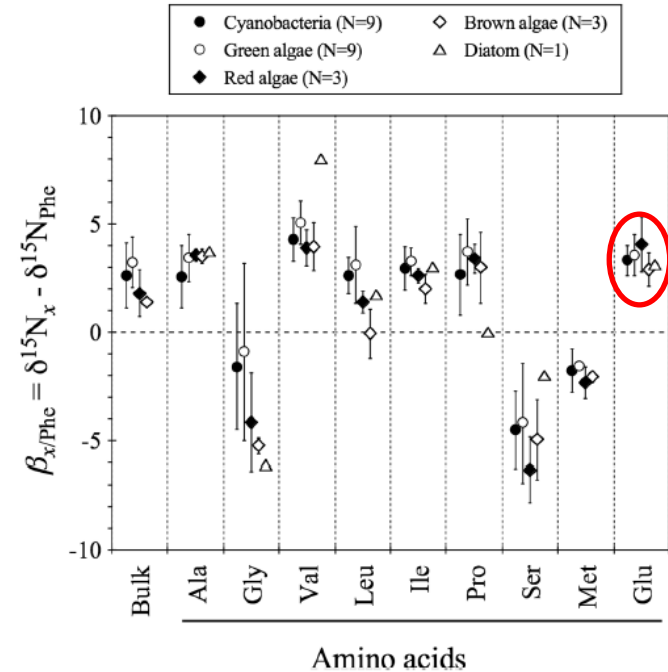
trophic position

¿why Glu & Phe?

	$\beta_{x,Phe}$		$\Delta$	
	Average	1 $\sigma$	Average	1( $\sigma$ )
Bulk	2.6	1.3	2.1	1.3
Alanine	3.2	1.2	6.1	2.1
Glycine	-2.3	3.4	3.7	3.9
Valine	4.6	1.2	5.0	1.7
Leucine	2.3	1.6	4.8	2.0
Isoleucine	2.9	0.8	4.8	1.7
Proline	3.1	1.7	6.1	1.6
Serine	-4.6	2.2	3.6	3.0
Methionine	-2.0	0.6	0.5	0.6
Glutamic acid	3.4	0.9	8.0	1.2
Phenylalanine	—	—	0.4	0.5



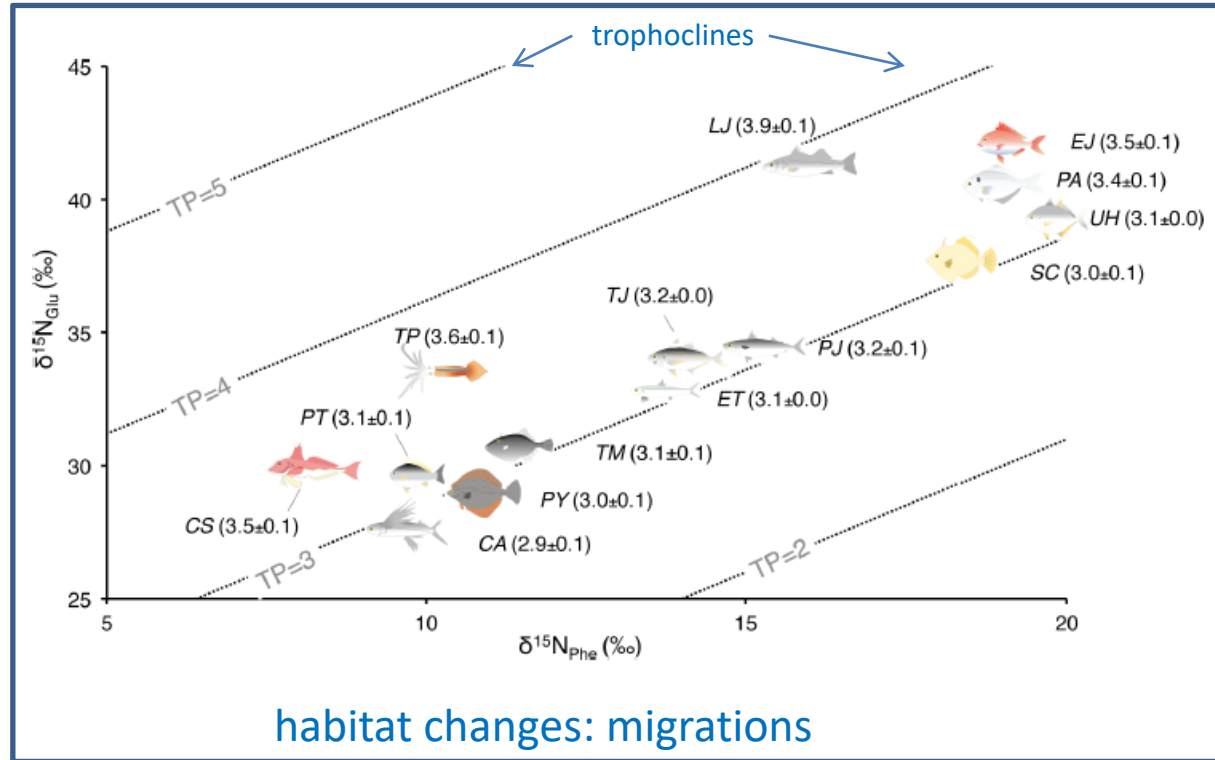
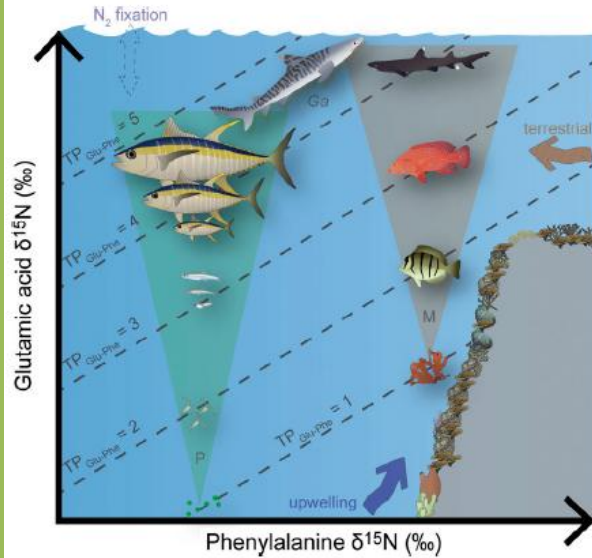
$\Delta_{Glu}$  highest discrimination  
 $\Delta_{Phe}$  lowest variability  
 } between trophic levels



$\beta_{Glu/Phe}$  less variable

# Applications

trophic position

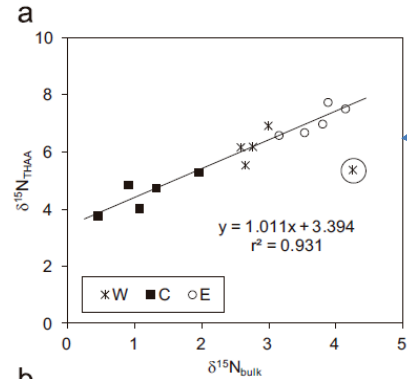


changes in the N source

habitat changes: migrations

# Applications

$\delta^{15}\text{N}_{\text{bulk}}$  vs.  $\delta^{15}\text{N}_{\text{CSIA}}$

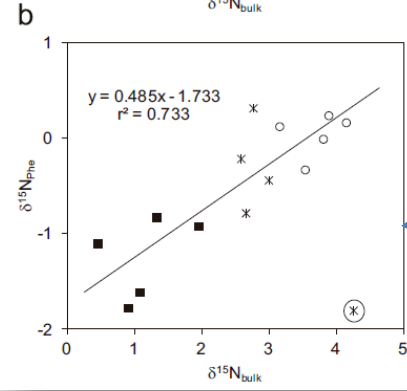


all AA

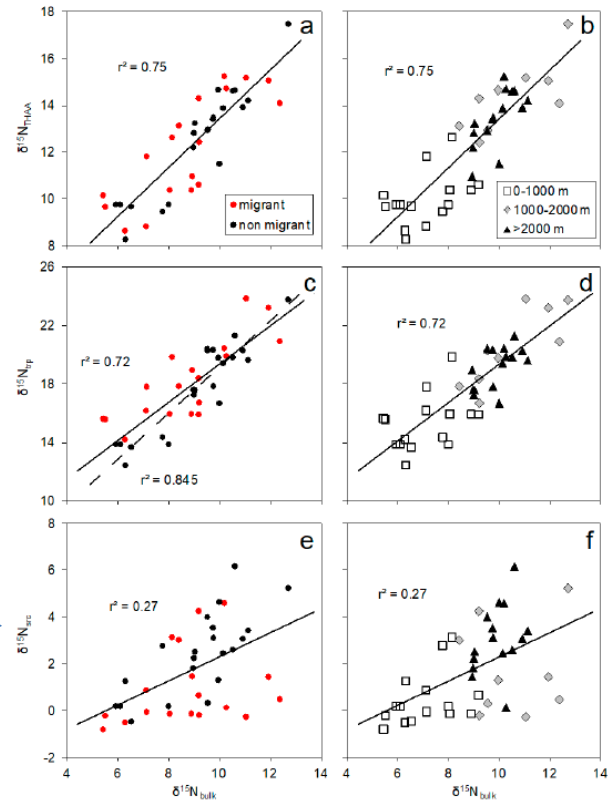
CSIA  $\cong$  bulk

trophic AA

source AA



$\delta^{15}\text{N}_{\text{bulk}}$



# Applications

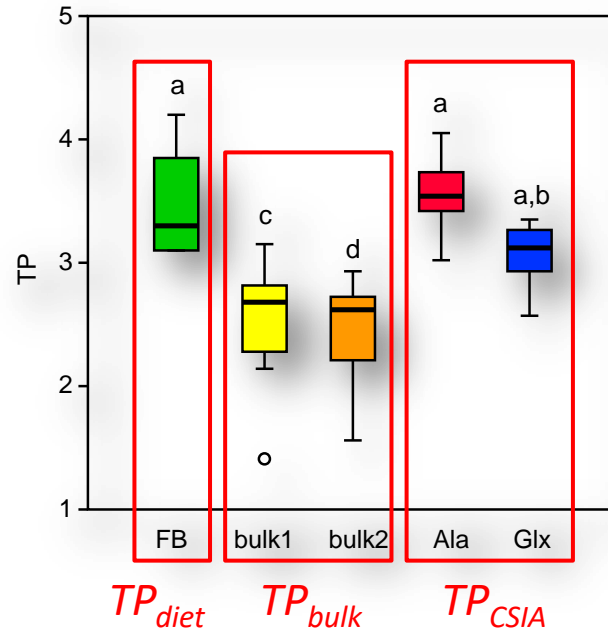
$TP_{bulk}$  vs.  $TP_{CSIA}$

Comparing different  $TP$  estimates

micronekton



Stomiiformes



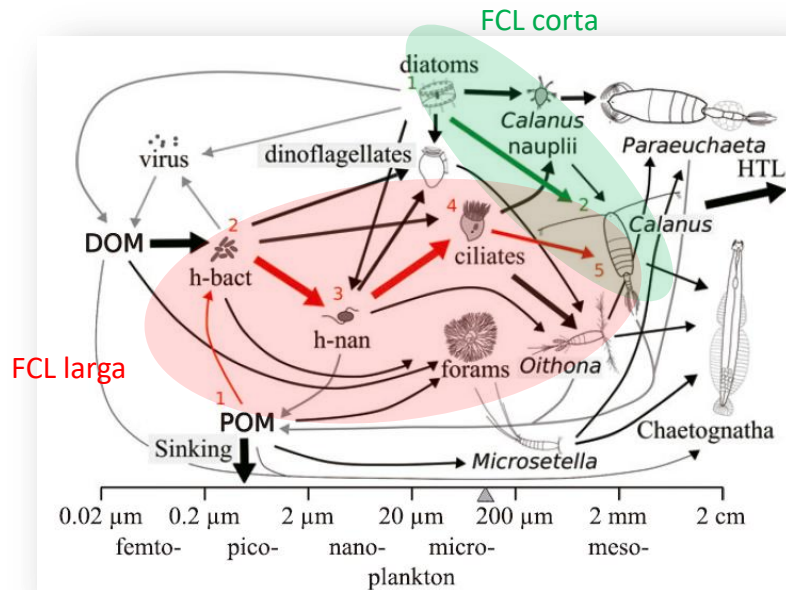
- food chain length =  $TP_{max}$
- heterotrophic resynthesis (degradation index)
- baseline identification = trophic systems
- diazotrophic contribution
- microbial system contribution
- diet indicators



# Estimation models

## food chain length (*FCL*)

$FCL = TP_{max}$  = number of trophic steps from primary producers to top predators

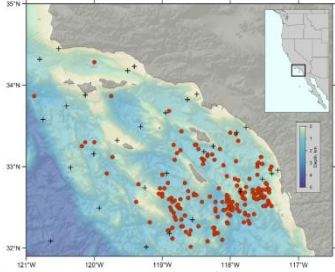


- food web dynamics
- trophic cascades
- relationships between diversity and function

# Application

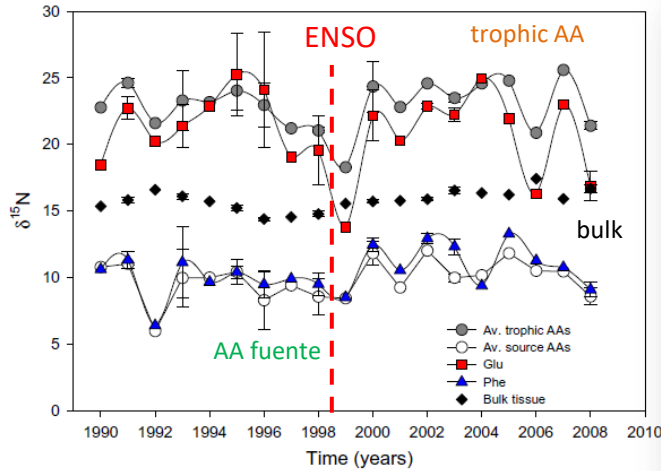
# food chain length

## California

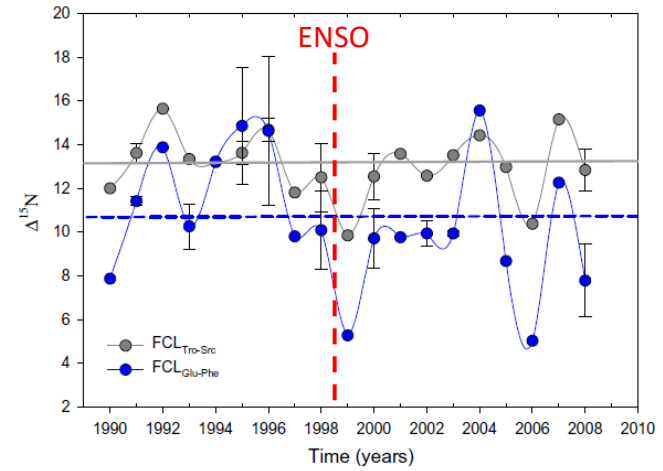


common dolphin

## $\delta^{15}\text{N}$ variability



## FCL ( $TP_{CSIA}$ ) variability



- ENSO affects trophic structure even in upwelling ecosystems
- $FCL_{max}$  in intermediate environmental conditions (temperature, chlorophyll, plankton)

# Estimation models

Heterotrophic resynthesis  
(degradation index =  $\sum V$ )

$$\sum V = \sum |\delta^{15}\text{N}_{\text{ti}} - \delta^{15}\text{N}_{\text{tm}}| / n$$

absolute value

number of trophic-AA

individual trophic-AA values

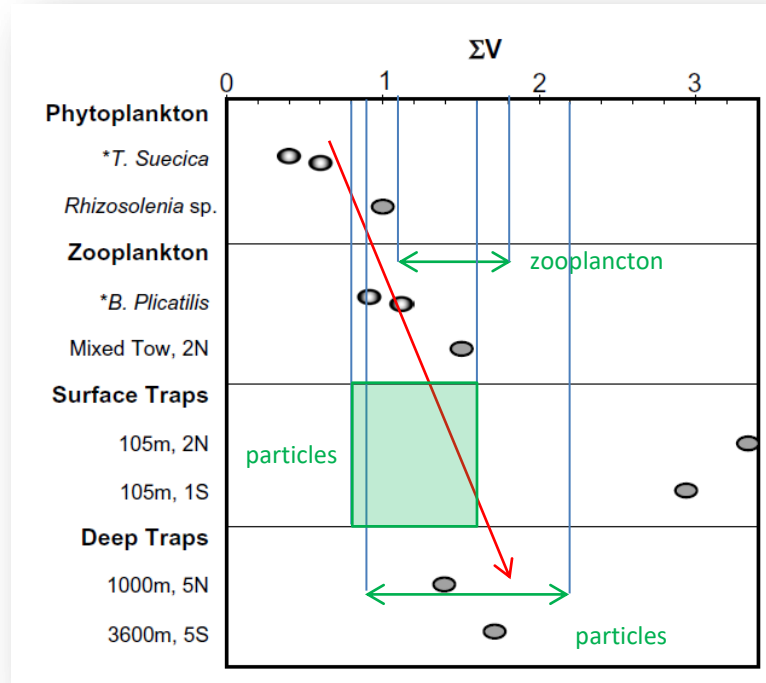
mean trophic-AA value

Ala, Asp, Glu, Ile, Leu, Pro

# Application

## Heterotrophic resynthesis

$\Sigma V$  in plankton vs. sediments



[\(Hannides et al., 2013\)](#)

[\(Mompeán et al., 2016\)](#)

increase of degradation by bacteria?

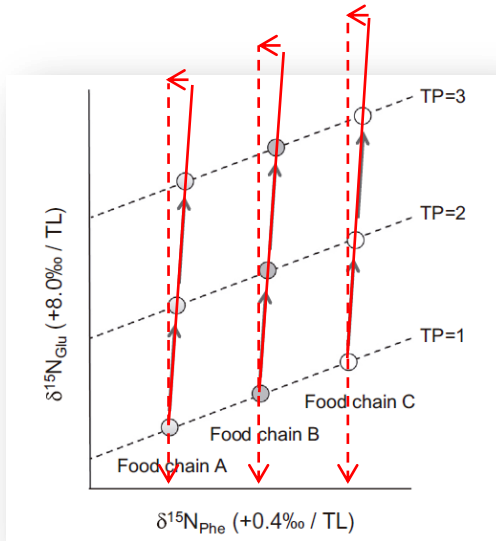
[\(Shen et al., 2021\)](#)

[McCarthy et al., 2007](#)

# Estimation models

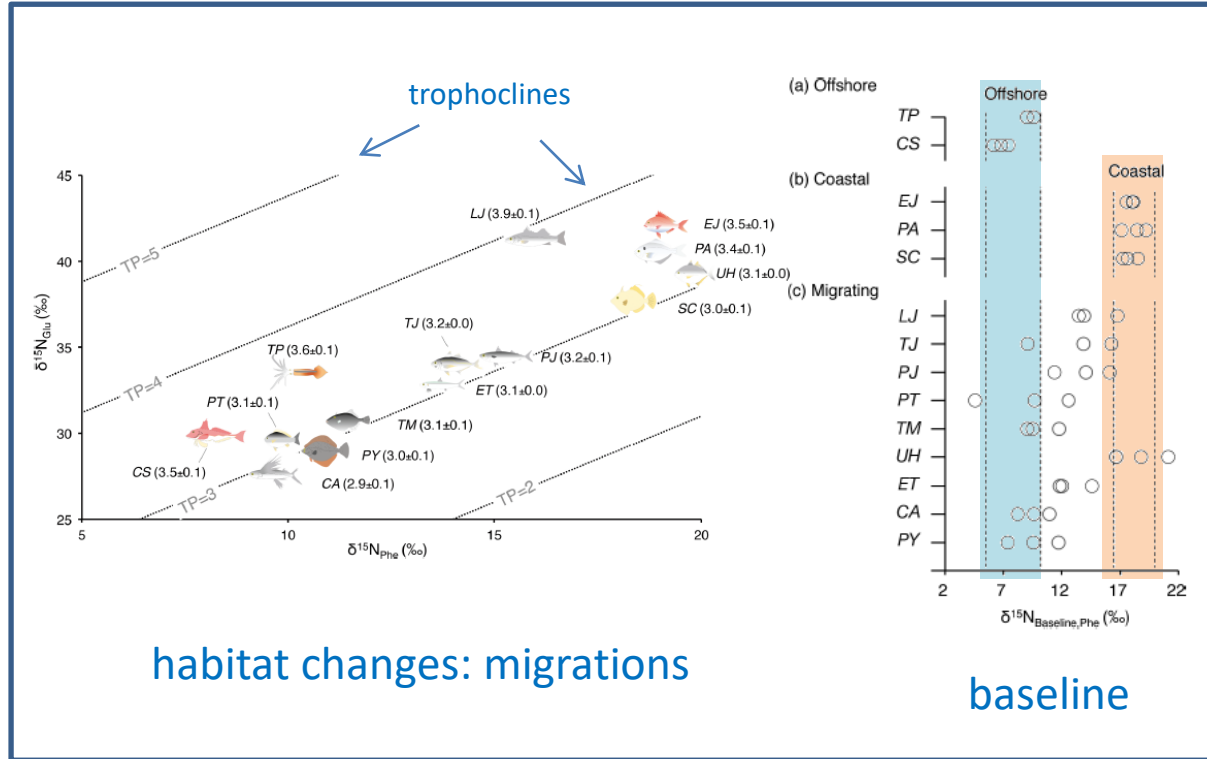
## baseline identification

baseline comparison for different TPs



$$\delta^{15}\text{N}_{\text{Baseline,Phe}} = \delta^{15}\text{N}_{\text{Phe}} - \beta_{\text{Phe}} (\text{TP} - 1)$$

+0.4‰



# Estimation models

## Diazotrophy contribution (%N<sub>fix</sub>)

BSIA

[Montoya et al., 2002](#)

$$\%N_{\text{fix}} = 100 (\delta^{15}\text{N}_{\text{bulk}} - \delta^{15}\text{N}_{\text{ref}}) / (\delta^{15}\text{N}_{\text{diazotroph}} - \delta^{15}\text{N}_{\text{ref}})$$

-2‰

sample

reference %N<sub>fix</sub> = 0

$$\%N_{\text{fix}} = 100 (\delta^{15}\text{N}_{\text{bulk}^*} - \delta^{15}\text{N}_{\text{ref}}) / (\delta^{15}\text{N}_{\text{diazotroph}} - \delta^{15}\text{N}_{\text{ref}})$$

CSIA

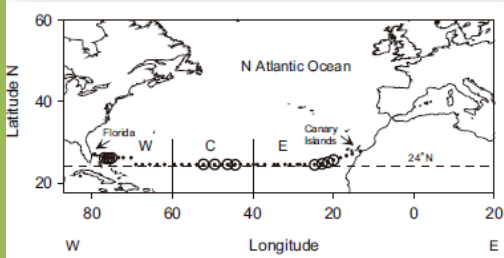
[Mompeán et al., 2016](#)

$$\delta^{15}\text{N}_{\text{bulk}^*} = \delta^{15}\text{N}_{\text{Phe}} + \beta_{\text{bulk/Phe}}$$

+2.7‰

# Application

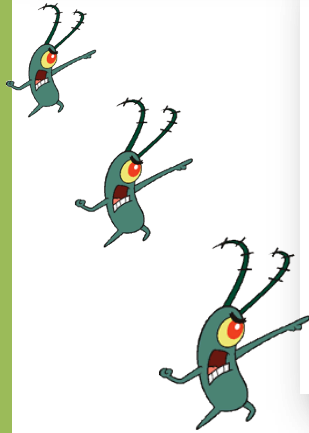
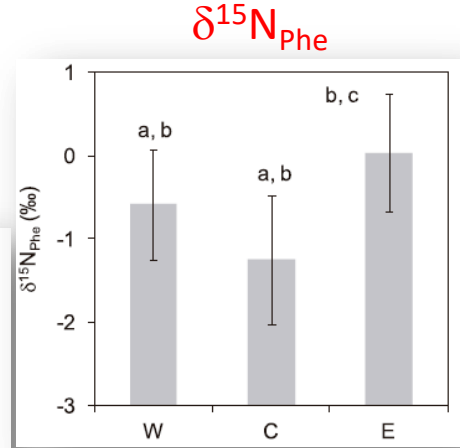
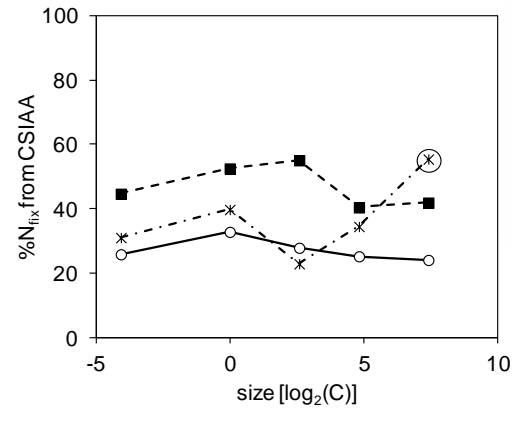
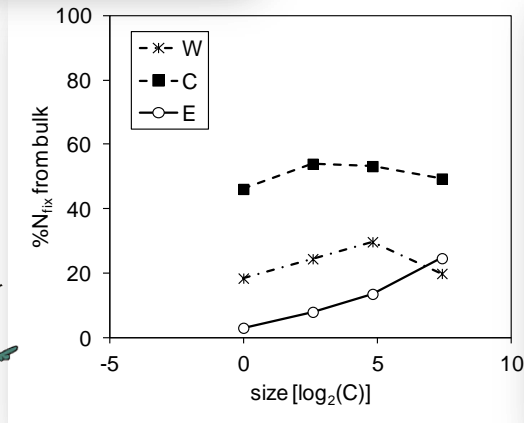
# Diazotrophy contribution



zooplancton size fractions

BSIA

CSIA



uniform diazotrophic contribution in all fractions

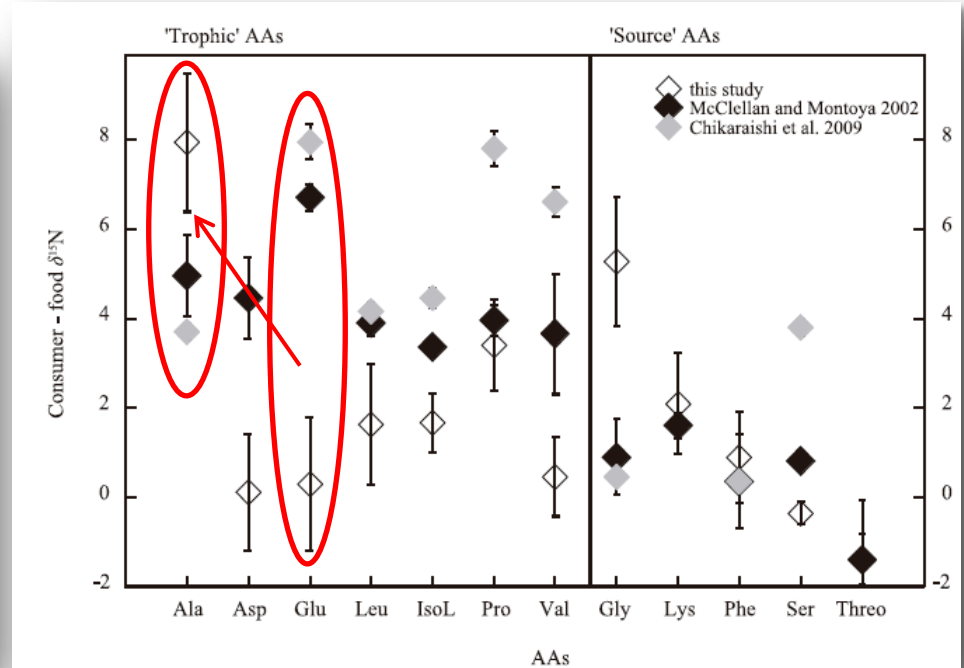
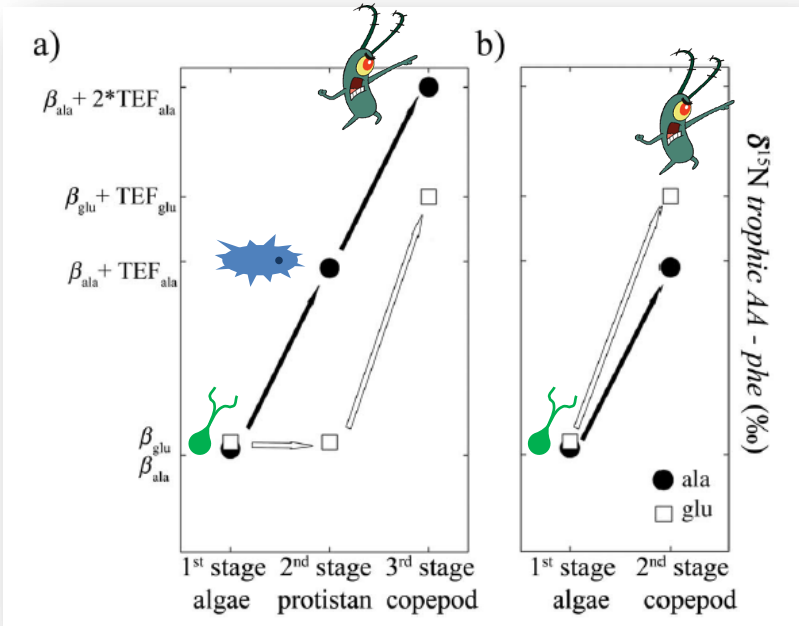
[Mompeán et al., 2016](#)



# Estimation models

microbial food web 'invisible' for  $TP_{Glu}$

microbial contribution to  $TP$   
(%microbial)



[Decima et al. 2017](#)

[Gutiérrez-Rodríguez et al. 2014](#)

# Estimation models

## microbial contribution to $TP$

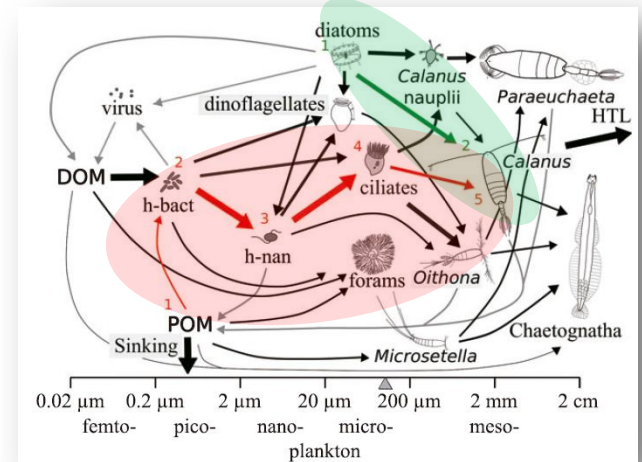
$$\% \text{ microbial} = 100 (TP_{Ala} - TP_{Glu}) / TP_{Ala}$$

TP microbial + metazoa

$$TP_{Ala} = 1 + (\delta^{15}N_{Ala} - \delta^{15}N_{Phe} - \beta_{Ala}) / TDF_{Ala}$$

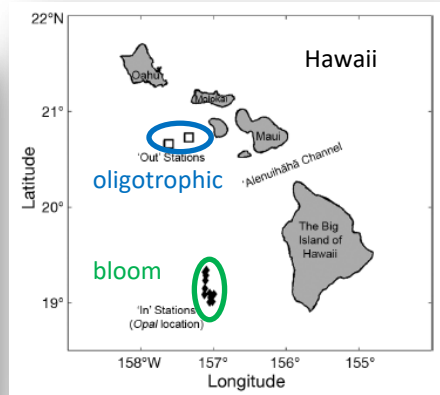
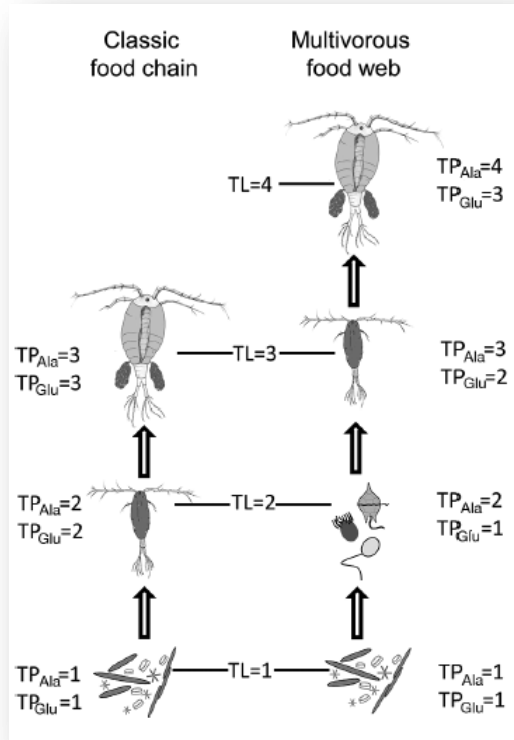
$$TP_{Glu} = 1 + (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - \beta_{Glu}) / TDF_{Glu}$$

TP metazoa



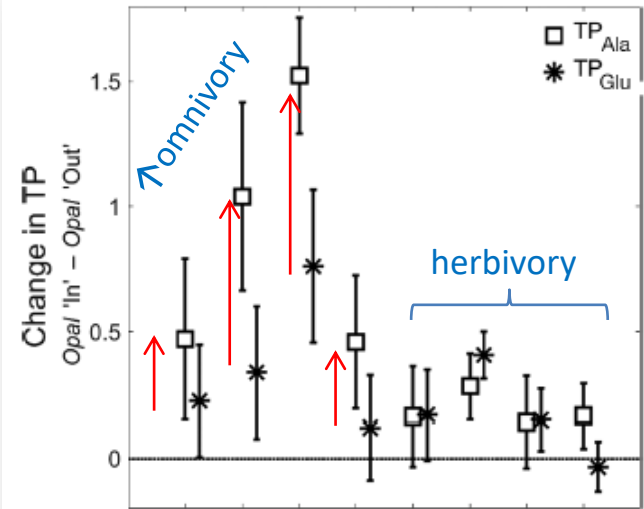
# Application

## microbial contribution to *TP*



increase of predation on protozoa  
in the bloom zone

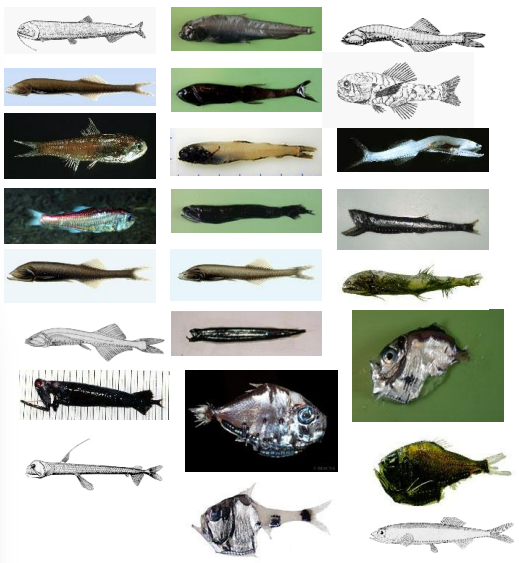
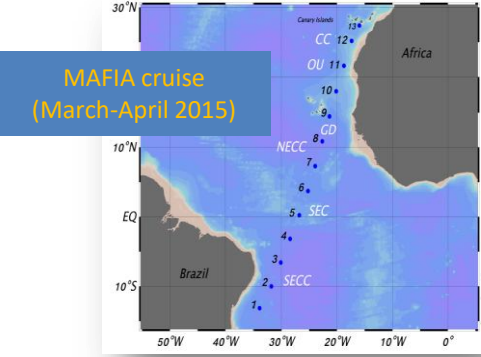
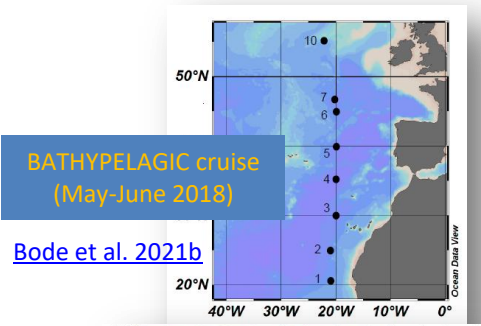
$$TP_{bloom} - TP_{oligotrophic}$$



# Application

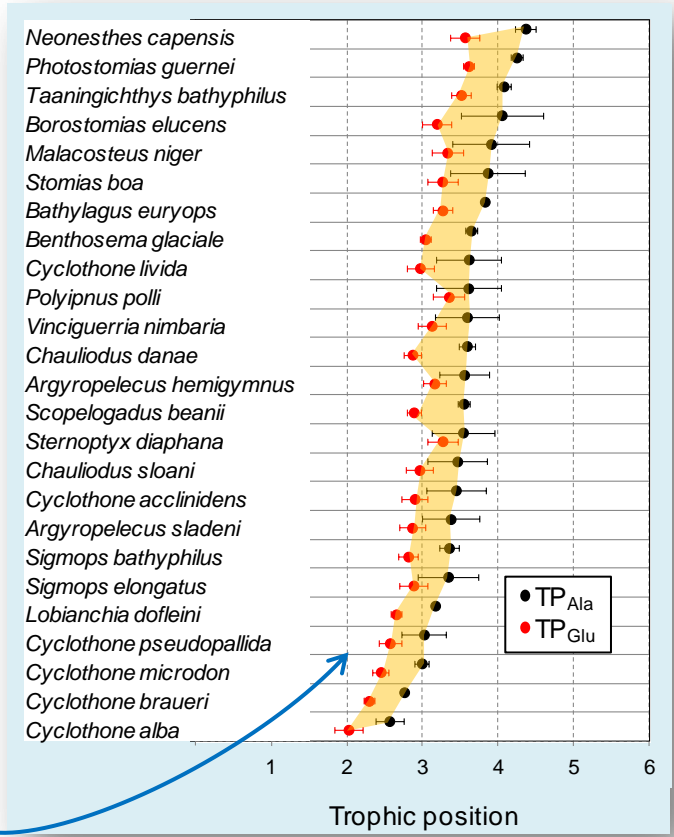
# microbial contribution to TP

## micronekton



$TP_{Glu}$  subestimates TP

%microbial = 6-21%

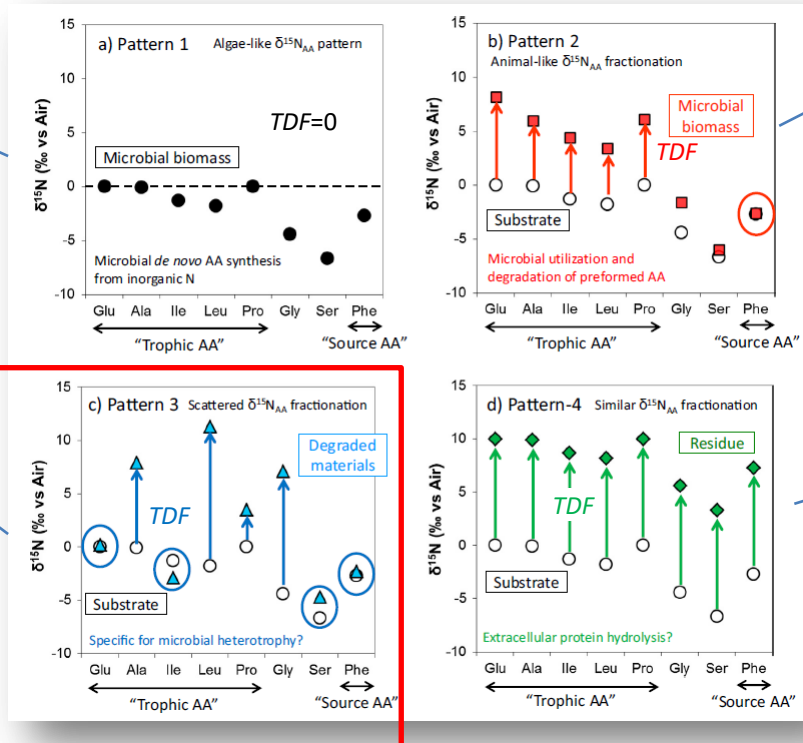


# Estimation models

# microbial contribution to *TP*

## TDF patterns in chemolithotrophic bacteria

pure cultures  
'de novo' AA synthesis



pure cultures  
use of high quality protein

microcosms  
direct incorporation of some AA (e.g. Glu) and resynthesis of other AA (e.g. Ala)

hypothesis  
extracellular hydrolysis  $TP = 2$  (e.g. PON of sedimenting particles)

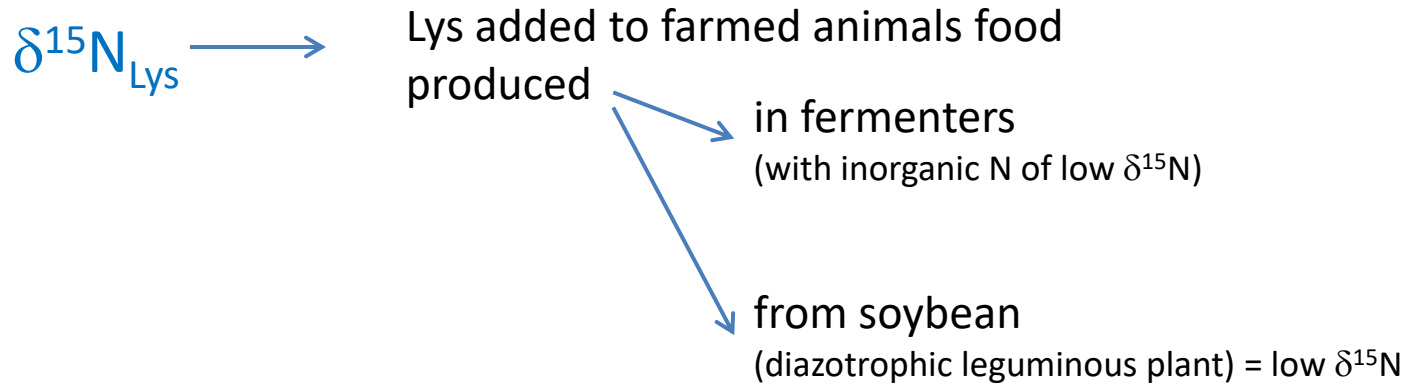
[Ohkouchi et al. 2017](#)

[McMahon & McCarthy, 2016](#)

# Estimation models

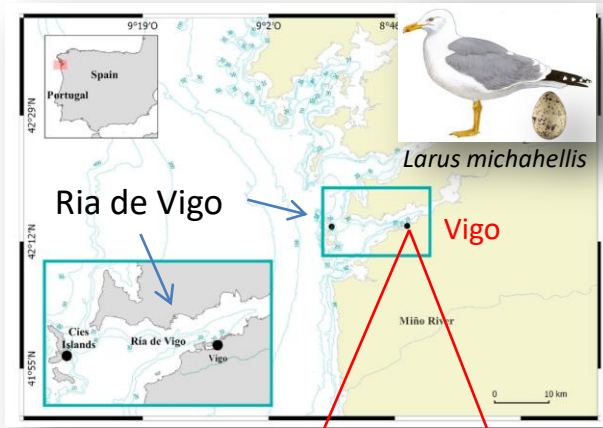
## diet indicators

example:

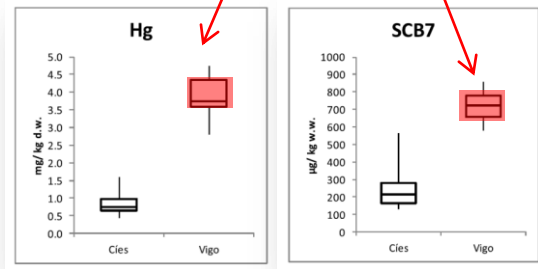


# Application

## diet indicators



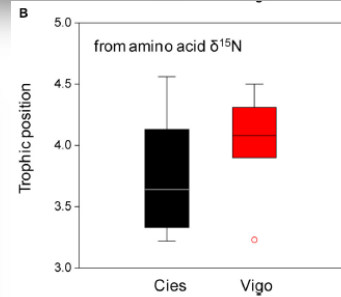
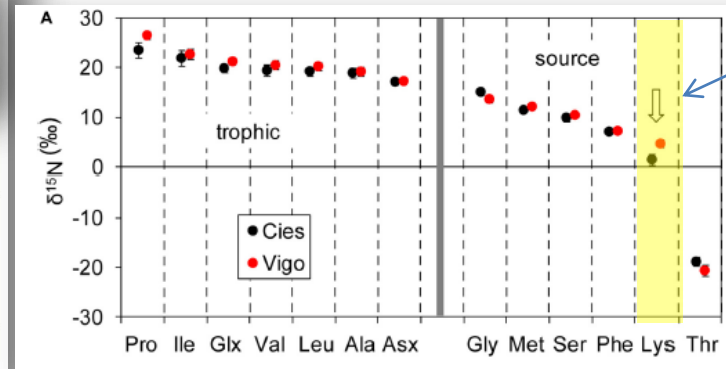
[Viñas et al. 2020](#)



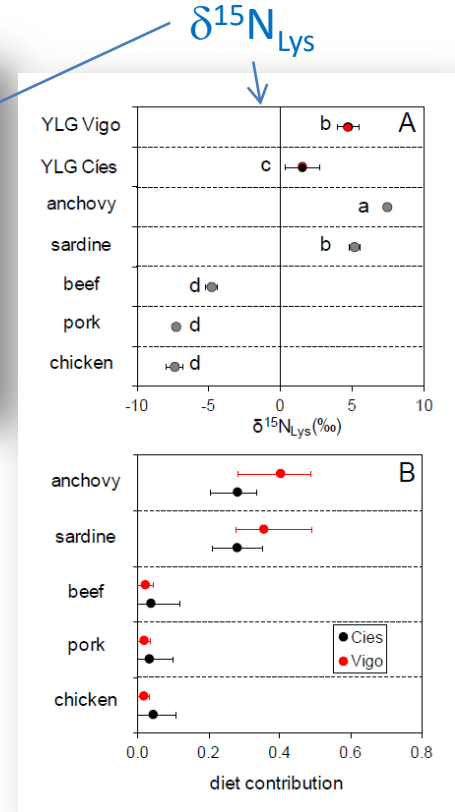
inorganic

pollutants

organic



same TP but different diet



[Bode et al. 2021d](#)

## Improvements:

$$TP_c = 1 + (\delta^{15}N_t - \delta^{15}N_s - \beta_{t/s}) / TDF_{t/s}$$

- multitrophic models
  - multi-TDF
  - multi- $\beta$
- error propagation

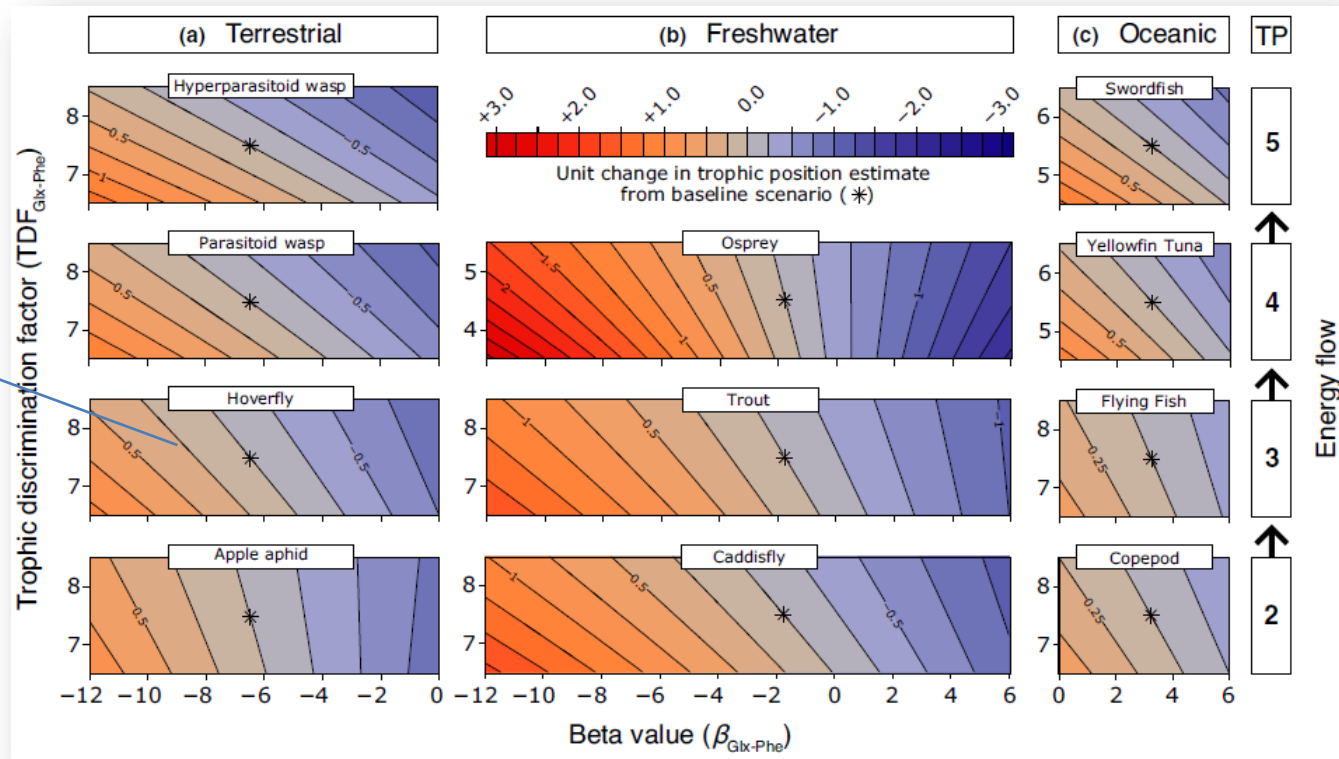


# Improvements:

# multi-TDF & multi- $\beta$ models

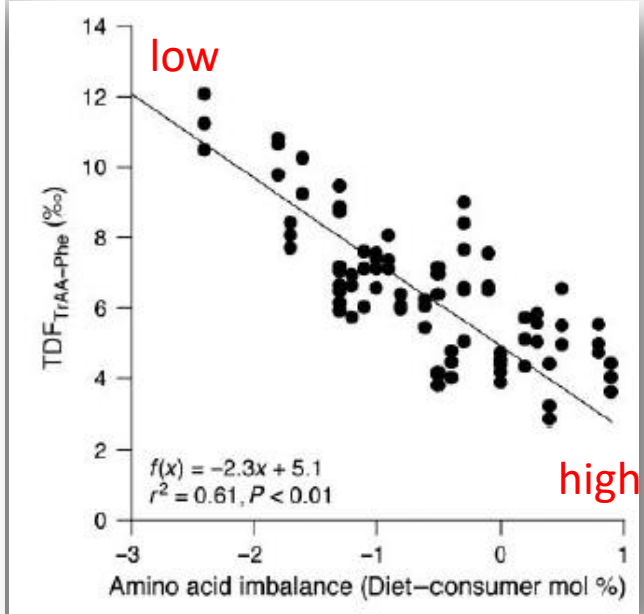
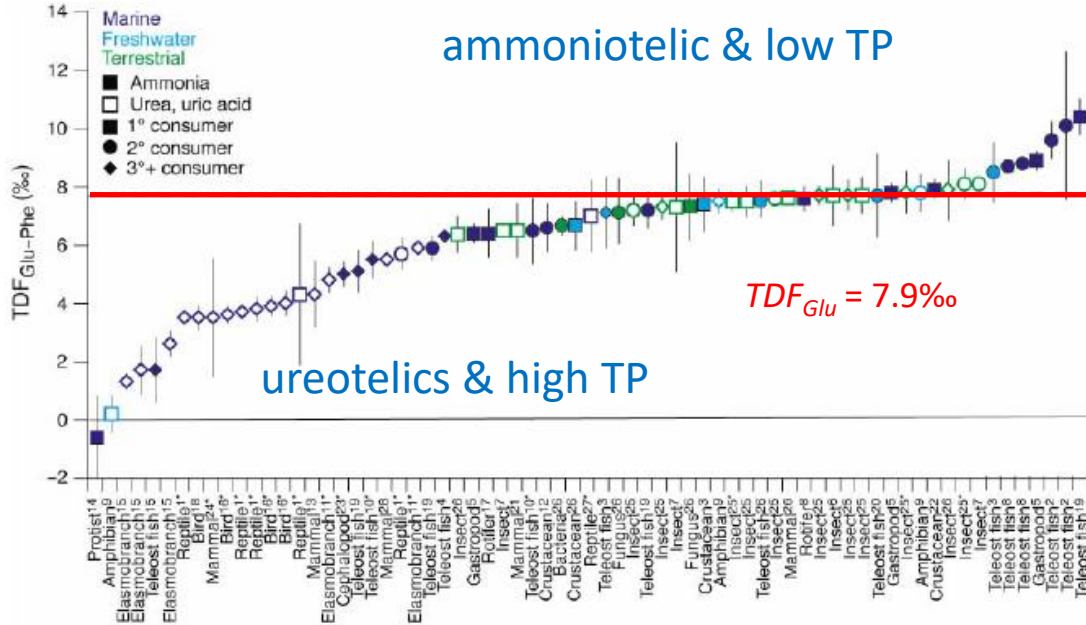
simulating variations in TDF and/or  $\beta$

TP variation



# Improvements:

## multi-TDF models

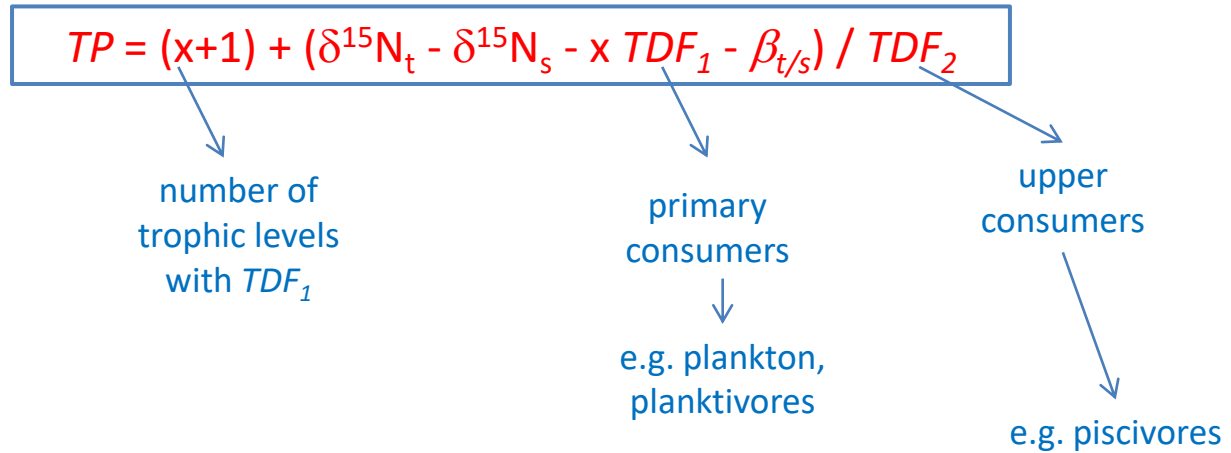


food quality

TDF varies with diet quality and N excretion modes

# Improvements:

## multi-*TDF* models



# Application

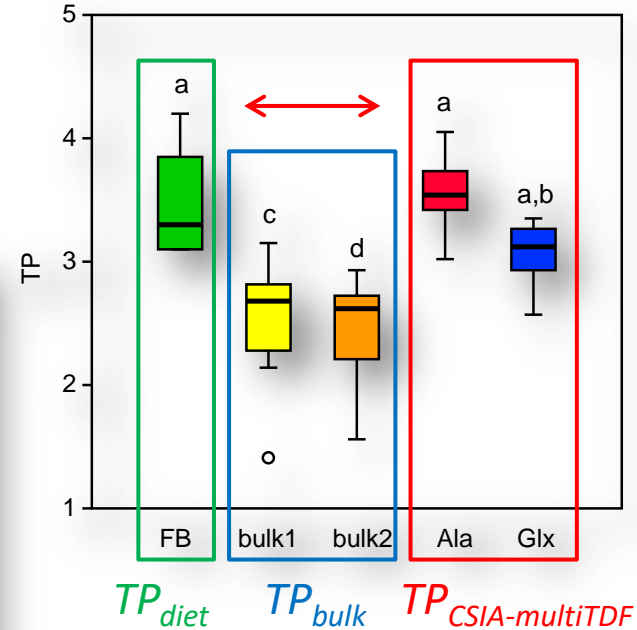
micronekton



Stomiiformes

Type	Equation	Parameters
Additive (bulk)	$TP_{bulk1} = \frac{(\delta^{15}N_s - \delta^{15}N_p)}{TEF_{bulk}} + 2$	$TEF_{bulk} = 3.4 \pm 1.0\text{‰}$
Scaled (bulk)	$TP_{bulk2} = \frac{[\log(\delta^{15}N_{lim} - \delta^{15}N_p) - \log(\delta^{15}N_{lim} - \delta^{15}N_s)]}{k} + 2$	$\delta^{15}N_{lim} = 2.93 \pm 0.71\text{‰}$ $k = 0.14 \pm 0.49$
Total (CSIA)	$TP_{Ala} = \frac{(\delta^{15}N_{Ala} - \delta^{15}N_{phe} - TEF_p - \beta)}{TEF_s} + 2$	$TEF_p = 4.5 \pm 2.1\text{‰}^\dagger$ $TEF_s = 6.1 \pm 0.3\text{‰}^\dagger$ $\beta = 3.2 \pm 1.2\text{‰}^\dagger$
Metazoan (CSIA)	$TP_{Glx} = \frac{(\delta^{15}N_{Glx} - \delta^{15}N_{phe} - TEF_p - \beta)}{TEF_s} + 2$	$TEF_p = 7.6 \pm 1.2\text{‰}^\dagger$ $TEF_s = 5.7 \pm 0.3\text{‰}^\dagger$ $\beta = 3.6 \pm 0.5\text{‰}^\dagger$

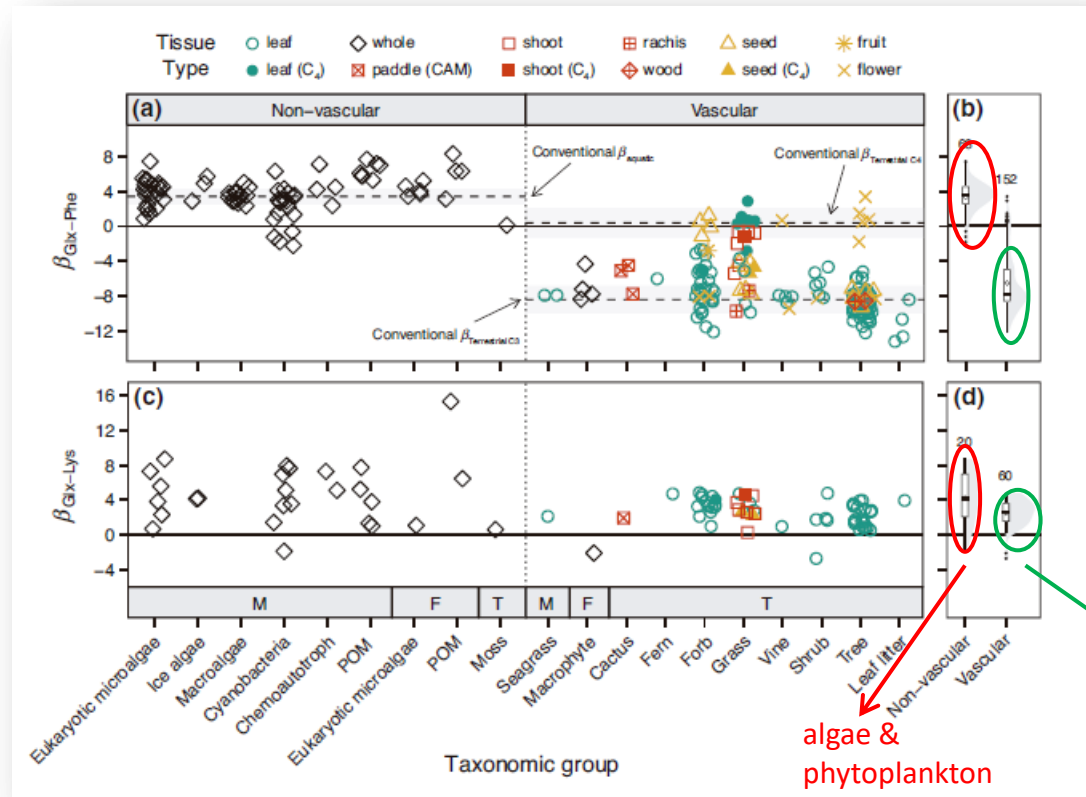
## multi-TDF models



more realistic TP with multi-TDF models

# Improvements:

multi- $\beta$  models

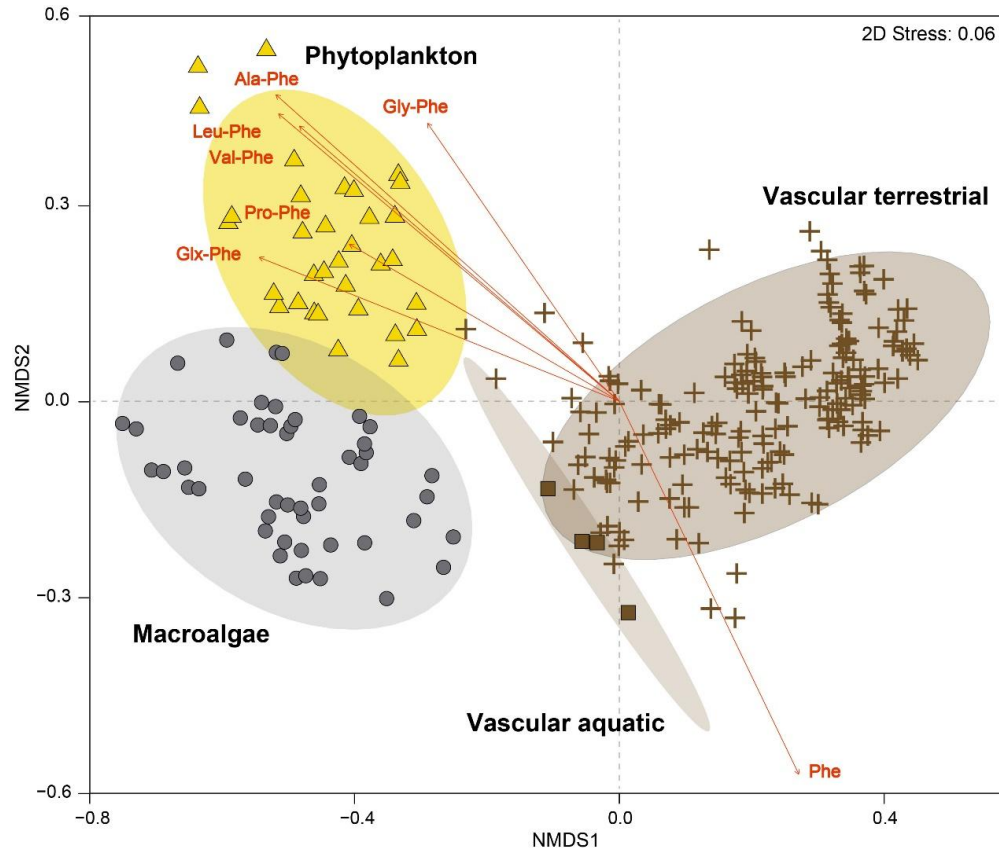


$\beta_{Glu-Phe}$

$\beta_{Lys-Phe}$

# Improvements:

multi- $\beta$  models



primary producer  
 $\delta^{15}\text{N}_{\text{AA}}$  patterns

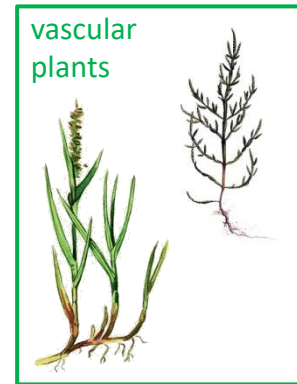
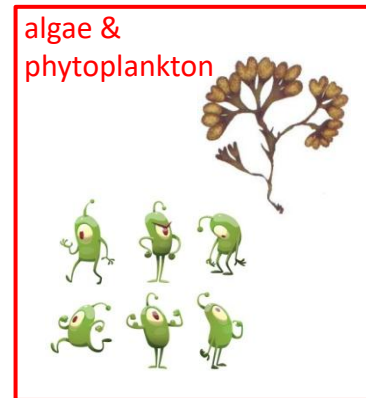
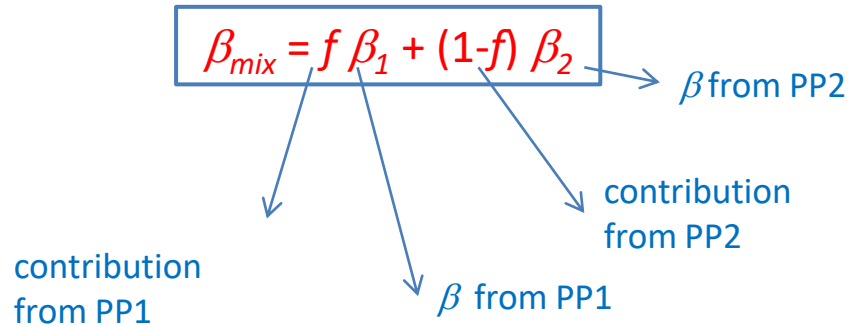
# Improvements:

## multi- $\beta$ models

When there are two primary producer (PP) sources

$$TP = 1 + (\delta^{15}N_t - \delta^{15}N_s - \beta_{mix}) / TDF$$

$$TP = (x+1) + (\delta^{15}N_t - \delta^{15}N_s - x TDF_1 - \beta_{mix}) / TDF_2$$



[Ramirez et al. 2021](#)

# Improvements:

$\delta^{15}\text{N}$ ,  $TDF$  and  $\beta$  measured with error

$$TP = 1 + (\delta^{15}\text{N}_t - \delta^{15}\text{N}_s - \beta) / TDF$$

Diagram illustrating the propagation of error from the measured variables to the Total Protein (TP) calculation. The equation is shown in red. Arrows indicate the flow of error propagation:  $\delta^{15}\text{N}_t$  and  $\delta^{15}\text{N}_s$  have blue arrows pointing to  $\pm sd$  labels above them.  $\beta$  has a blue arrow pointing to a  $\pm sd$  label to its right. The entire equation has a red arrow pointing to a  $\pm sd$  label below it. The  $TDF$  term has a blue arrow pointing to a  $\pm sd$  label below it. The difference term  $(\delta^{15}\text{N}_t - \delta^{15}\text{N}_s - \beta)$  has a blue arrow pointing to a  $\pm sd$  label below it.

propagated error of TP

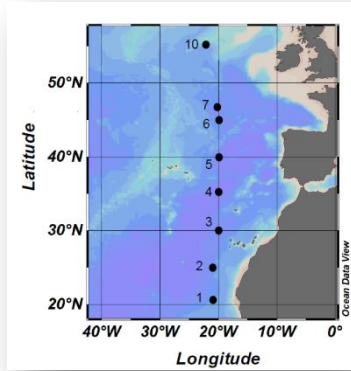
Montecarlo simulations

Taylor series expansion

$$sd_{TP}^2 = (1/TDF)^2 sd_{\delta^{15}\text{N}_t}^2 + (-1/TDF)^2 sd_{\delta^{15}\text{N}_s}^2 + (1/TDF)^2 sd_{\beta}^2 + [-1/TDF^2 (\delta^{15}\text{N}_t - \delta^{15}\text{N}_s + \beta)]^2 sd_{TDF}^2$$

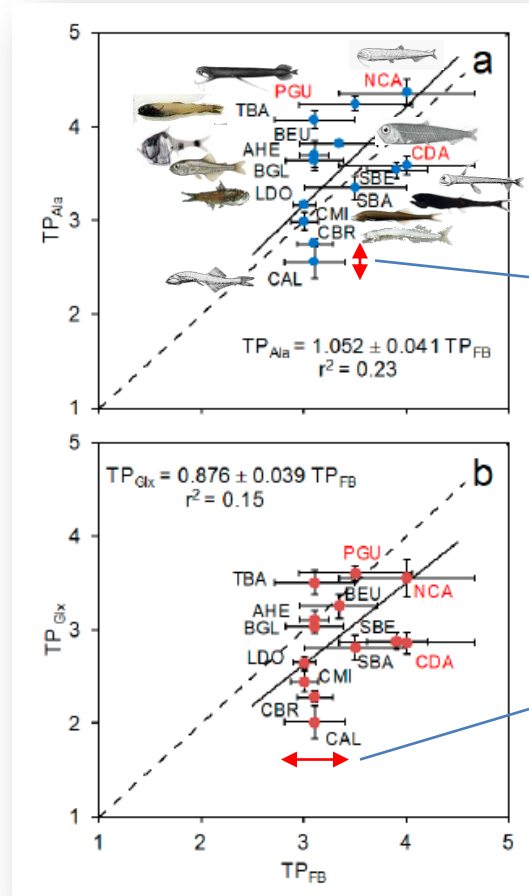


# Application



micronekton

*TP* propagated error



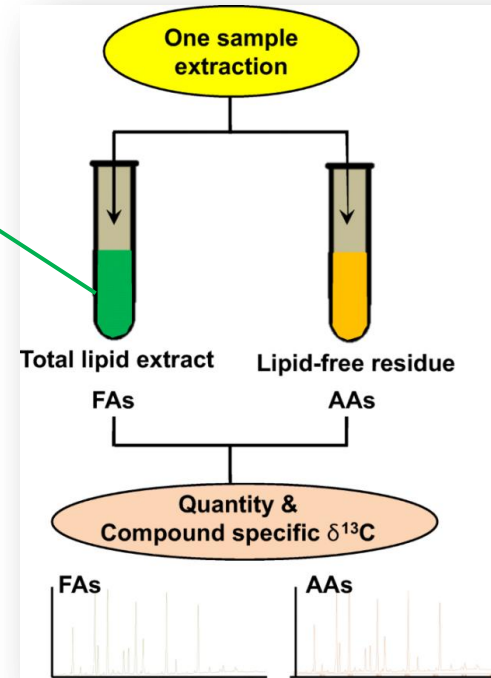
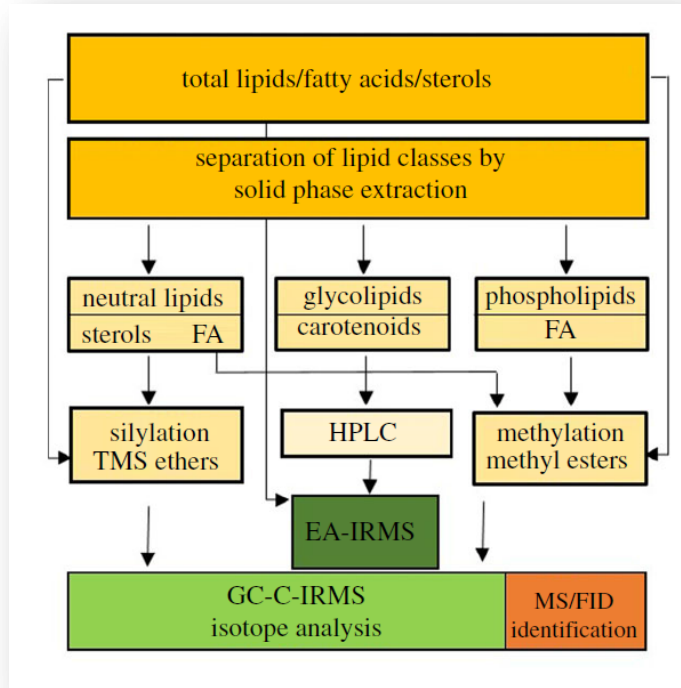
lower sd  $TP_{CSIA}$

higher sd  $TP_{diet}$

# Diet identification

carbon in:

- amino acids
- fatty acids

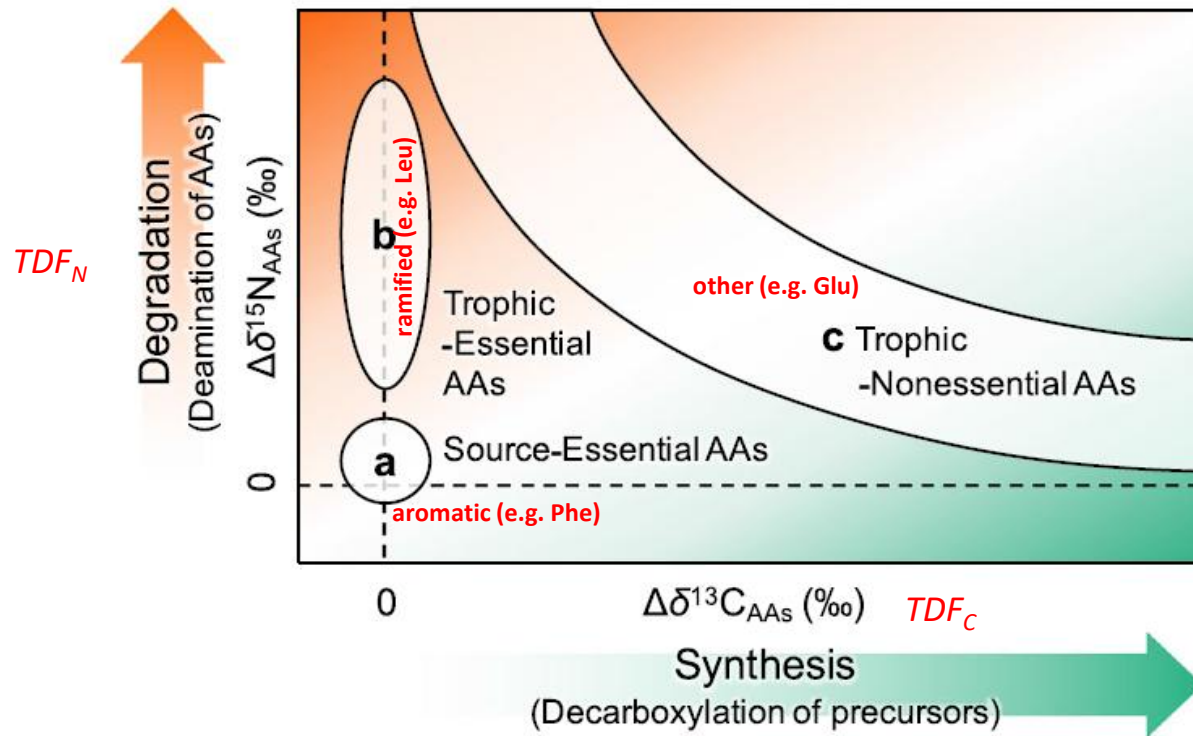


[Twining et al. 2020](#)

[Yun et al., 2020](#)

# Diet/habitat identification carbon and nitrogen in amino acids

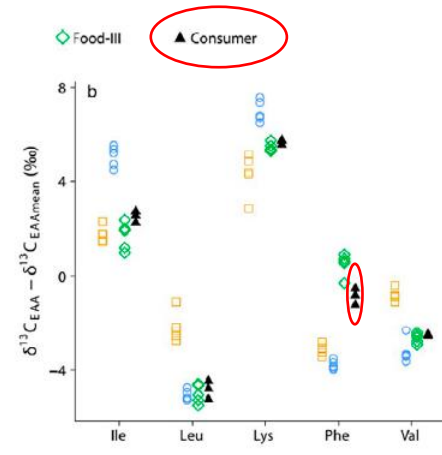
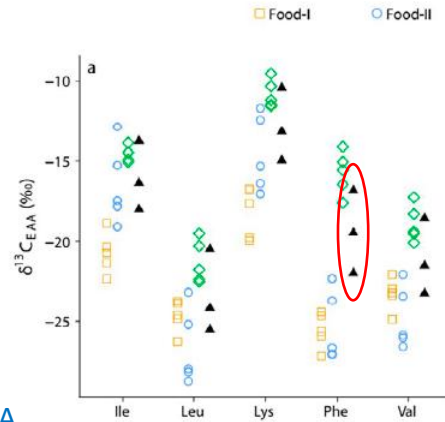
AAs grouped by C or N TDFs



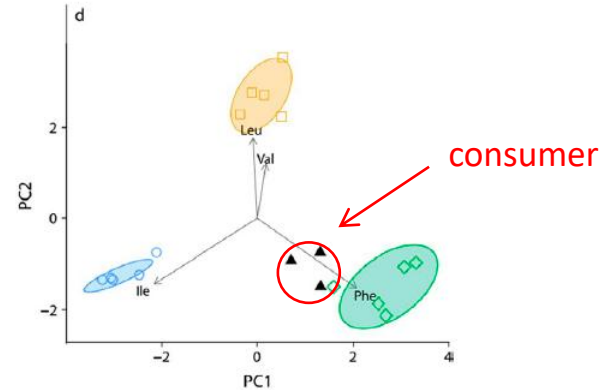
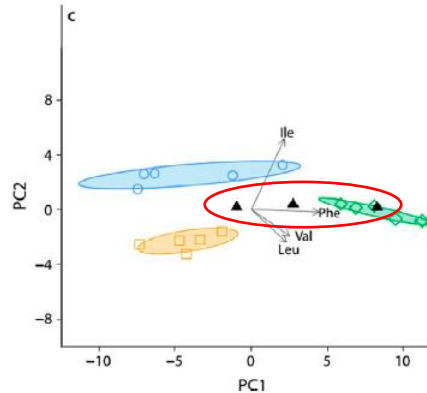
# Diet/habitat identification

## C in essential amino acids

non-centered  $\delta^{13}\text{C}_{\text{EAA}}$

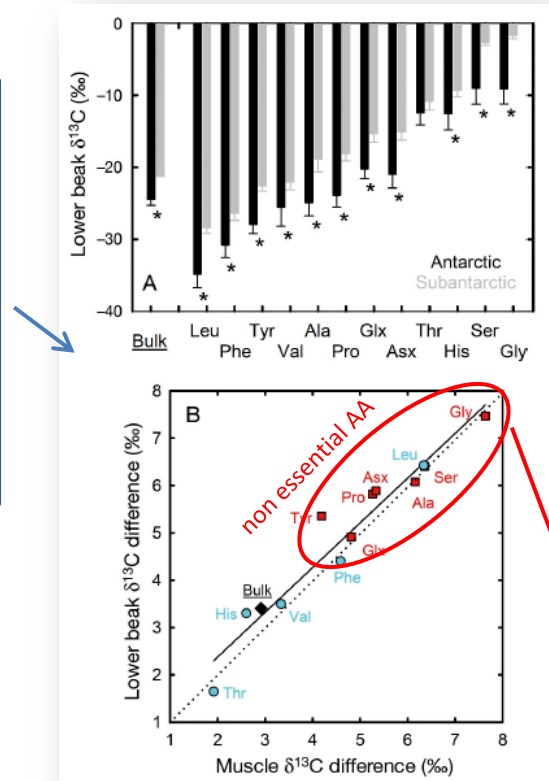
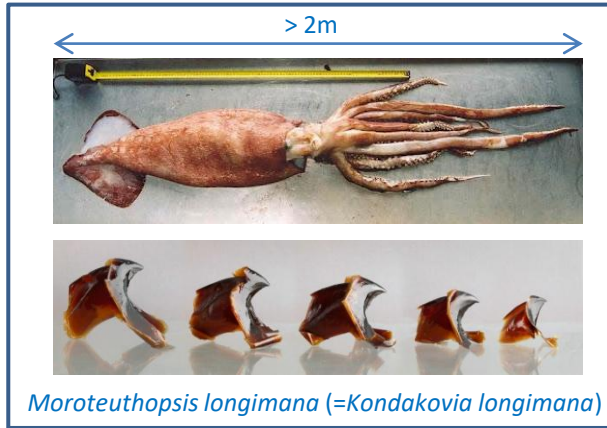


centered  $\delta^{13}\text{C}_{\text{EAA}}$



# Application

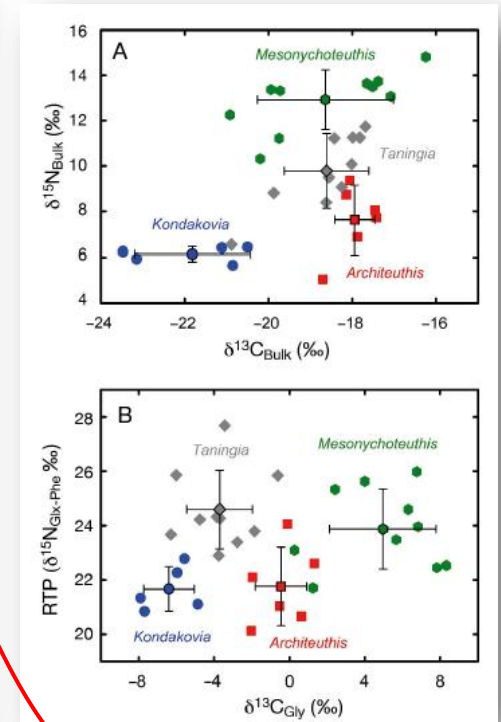
giant squids



$\delta^{13}\text{C}_{\text{AA}}$  used for habitat identification

[Cherel et al. 2019](#)

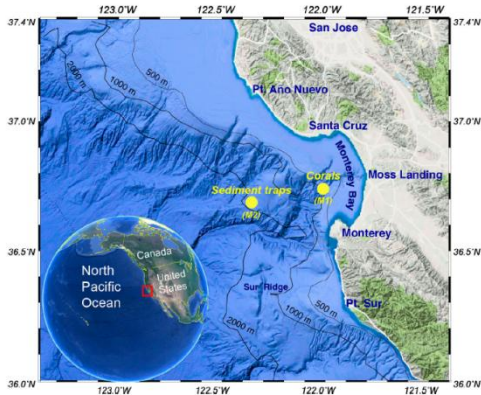
C in amino acids



$\delta^{13}\text{C}_{\text{Gly}}$  used for habitat-specific grouping

# Application

soft-corals and sinking particles

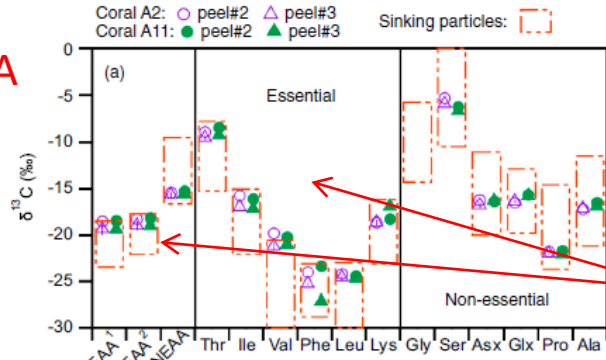


*Isidella* sp.

[Shen et al. 2021](#)

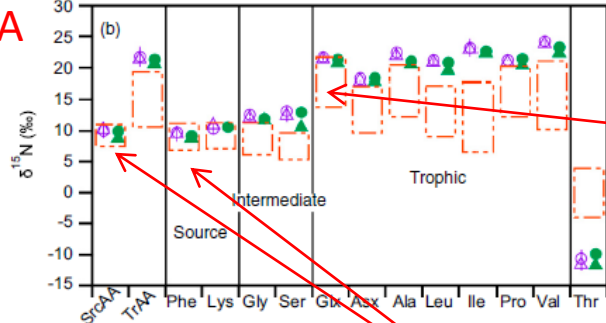
## C and N in amino acids

C-AA



reconstruction of:  
export production  
location offset

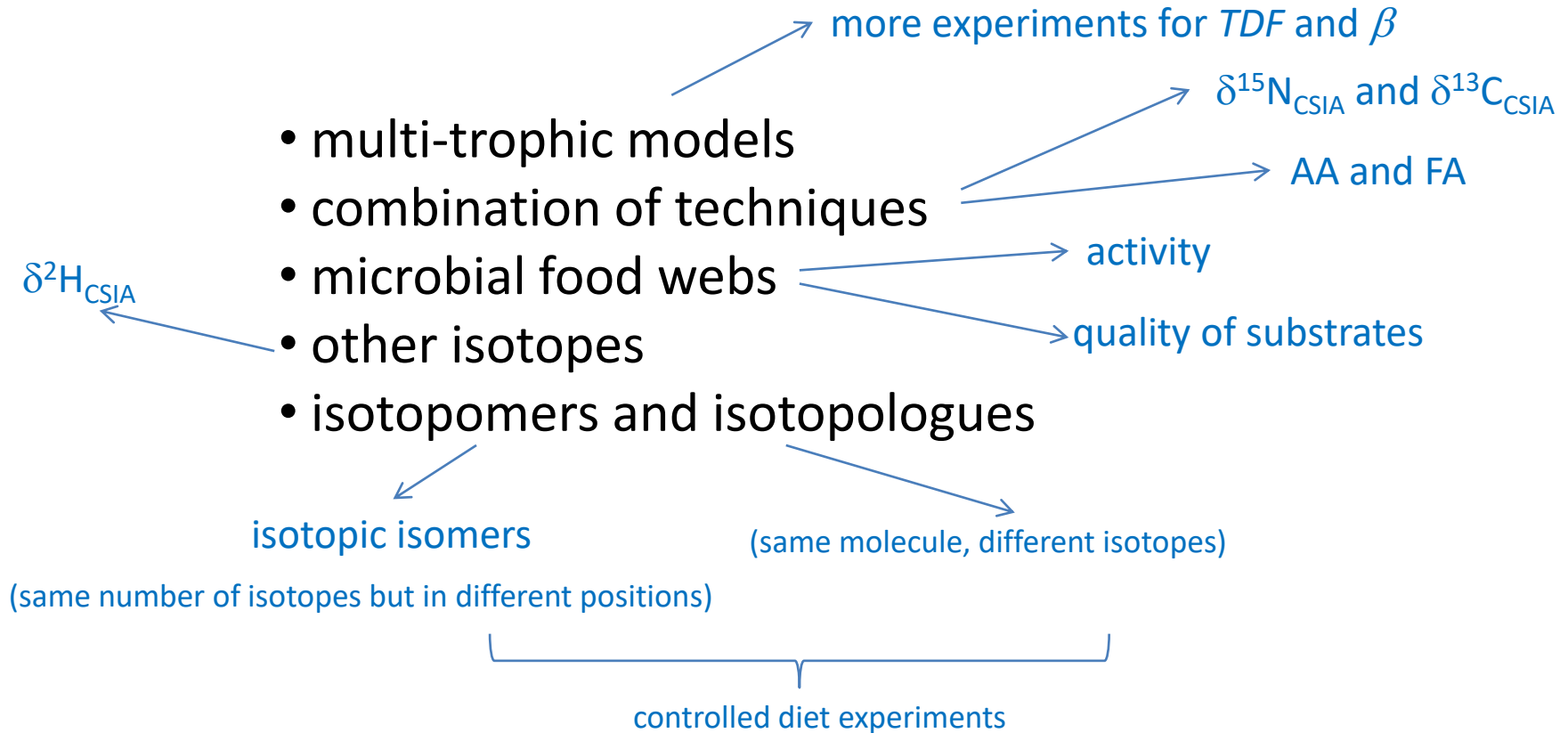
N-AA

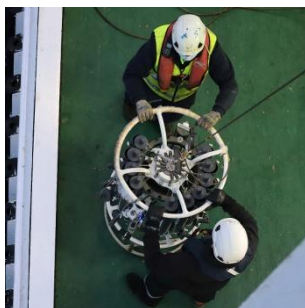
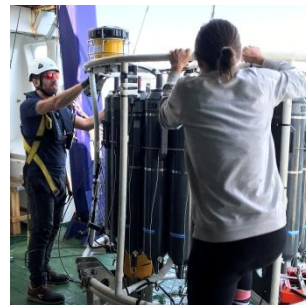


exported TP  
tissue offset

source nitrogen  
TP offset

# Future developments:





 **CSIC**  
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS



Grupo de investigación  
**Ecología Planktónica y Biogeoquímica (EPB)**

IEO-CSIC Centro Oceanográfico de A Coruña

