Compound-specific amino acid N-fractionation patterns:

*A new approach to organic nitrogen source and transformation*

With spin-offs from harbor seal health and Arctic warming, to Deep Sea Corals..
Co-Authors and Collaborators

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Background-1:
Most AA only have a single N

\[
\text{R} - \text{NH}_2 - \text{OH}
\]
Most AA only have single N
Practical implication: del 15N-AA patterns are much harder to measure than carbon.

• Less N: 1/3 to 1/10 as much (vs. C) to work with
• Less sensitivity in MS (ionization efficiency is LOWER)

→ need at least 10x more sample – and chromatography is very challenging
A few exceptions w/ multiple N- but we reliably get Lys – sometimes Arg

Lys: 2 N (amine)

Arg 3 N’s

His 3 N
Background-2:
lots of N flux in an organism
High (Remarkable?) degree of N flux through AA- meaning the abundant AA’s have very high flux
Background 3:

That single Amine N comes from SAME pathway and same source pool.
This means that:

1) Gln pool is source of most N in all other AA’s

1) In theory.. Glx that we measure (= Gln + Glu) should approximate source
Also in theory
Should mean:

1) Pattern should be very simple

2) All AA del 15N of amine N should look about like Glx?
Result: N patterns look quite different vs. C
δ^{15}N-AA patterns much more uniform

**DUE mostly one N, via transamination Rx from Glutamate pool**

*McClelland and Montoya, 2002*
But does it match expectations?

Sort of.. But for only about half of them
AA fall into two main groupings relative to Glx

Fundamental split in N metabolism between the 2 groups

Glx signal may be retained in the “non-fractionating” AA
But does it match expectations?

Group 1: matches expected (known) pathways

Group 2: always depleted—consistent with further processing?
But wait:

A terrible admission-

These two groupings were NOT our idea- and in fact were NOT published in relation to biochemistry.
A Step back: what do we “usually” use N isotopes for in organics?
**Basic use for N isotopes:** light isotope is preferentially enriched in N excreted by ANIMALS (ammonia).

"you are what you eat + 3 "

*Fig. 2. \( \delta^{15}N \) of excreted ammonium and bodily tissue for zooplankton collected at Stas B, C and F. (○) *Neocalanus cristatus* CV females, Sta. B; (■) *N. plumchrus* CV females, Sta. C; (▲) mixed calanoid copepodites and (●) *Diliolum denticulatum*, Sta. F. In total, \( \delta^{15}N \) was measured for 29 samples of bodies and 16 samples of excreted ammonium. Means, ranges, and Bartlett’s linear regression (\( \delta^{15}N_{\text{excreted ammonium}} = 0.96 \delta^{15}N_{\text{body}} - 2.7, r = 0.96, n = 7, P < 0.01 \)) are shown.*

Checkey, 1989- DSR
If AA are MOST ON, what pattern shift would one expect on Trophic Transfer?
The odd thing about N..

This doesn’t happen.
McClelland and Montoya, 2002: 
Differential processing of individual AA with Trophic Transfer

Enriched with trophic transfer

Not enriched with trophic transfer
Observations/hypotheses:

- **Phe retains its $\delta^{15}N$ value** best at base of food chain.
- **$\delta^{15}N$ offsets** (e.g. Glu – Phe) represents internally normalized record **trophic transfers**.

**Note:** groups do **NOT** correspond to “essential vs. non-essential” AA’s

- carbon skeleton metabolism not directly linked to N transamination..(?)
This two group “observational”- based on behavior w/ trophic transfer has become one of THE hot topics in isotope ecology

Other studies found similar behavior in diverse organisms:
• Hare et al. (1991) – pigs, whales, zebras
• Popp et al. (2007) – tuna
• Hannides et al. (2009) - zooplankton
But: nobody really understands it. Ie: why does this happen?
An Hypothesis: there two categories match pretty closely- and are NOT an accident...

**Group 1:** matches expected (known) pathways = TROPHICS (mostly)

**Group 2:** depleted- consistent with further processing? = “SOURCE”
Two basic possibilities:

1. **Additional biochemical pathways** cause depletion in “source” AA- **AND** these pathways are not conserved (or not used often) higher animals
   - this would make the “source” AA’s, truly analogous to “essential” AA’s.

2. **Somehow Related to relative Flux**- “Trophics” are AA’s with high concentration and N Flux- so they always ~ match Glu pool. “Source” have lower fluxes- so they can depart from the Glu pool. (but why always depleted??)
IF it was the second (seems more reasonable)

should be able to predict grouping or behavior based on mol%
Major sources of Proteinaceous material look pretty similar

After Cowie and Hedges, 1992
N- “Trophics”:
Ile, Leu, Val - Ala, Asp, Glu & Pro

• 3 of 4 of the “big 4” AA’s (non-Essential C) – *Gly is missing*
* The 3 aliphatic chains - Essentials
+ Pro!

* All “major” or “moderate: abundance- NO minor AA’s
Problem: if you try to use mol% to predict fractionation away from Glx-
it doesn’t work.  
(Lehman & McCarthy, in prep) 

Must be something else going on
A sort of mixed suggestion:
chikarashai et al. 2007, MEPS
(figure reproduced cryptically in 2009, L&O methods )

**Idea:**
- “dominant” catabolism pathway of some (Met, Phe) AA’s does not involve transmutations- therefore **no fractionation**

- **Problem:** explains only two AA- does not explain the “two groups

- **Better component:** Nod to idea that fractionation will depend on **both flux and enzymatic delta value**-
Anyway.. No one exactly knows yet.

But there are many exciting possibilities from these patterns.
1) “true” trophic level estimates, independent of del 15N in environment.

Solves one of most basic problems in N isotope systematic- is signal you measure due to source 15N, or trophic transfers?
The “Sashimi project” (Popp labs, UH)

Insight into the Trophic Ecology of Yellowfin Tuna, Thunnus albacares, from Compound-Specific Nitrogen Isotope Analysis of Proteinaceous Amino Acids

Brian N. Popp, Brittany S. Graham, Robert J. Olson, Cecelia C. S. Hannides, Michael J. Lott, Gladis A. Lopez-Ibarra, Felipe Galván-Magaña, and Brian Fry
Insight into the Trophic Ecology of Yellowfin Tuna, Thunnus albacares, from Compound-Specific Nitrogen Isotope Analysis of Proteinaceous Amino Acids

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In: Isotopes as Tracers of Ecological Change
Change in del 15N across the basin: Tropic shifts or tied to migration/location?
Latitude shifts in bulk plankton (~ 8 per mil from 8 S to 24 N) also observed in bulk tuna.
However, two interpretations possible:

1. Trophic structure (or small tuna feeding habits) changing.

2. Base autotroph 15N values are changing
Concept:
Source AA vs. Trophic-AA

![Graph showing tuna change in trophic level and feeding location](image)

- **A: Tuna change trophic level**
  - Enrichers
  - Bulk tuna
  - Non-enrichers

- **B: Tuna change feeding location**
Phe tracks Asp- suggests feeding at same trophic level across basin
2) *Phe* (and others?) may record original plankton 15N at base of food webs.

Useful in many, many areas of oceanography and ecology!
2) Deep Sea Corals as “living sediment traps”
15N-AA tracks: 15N level of primary production and tropic information back in time;

13C-AA in essential AA’s: 1) 13C of agal production (DIC), 2) algal sources?
Changes in isotopes of POM in Nova Scotia current in last ~ 100 yrs- why?
Data: beautiful

suggests strong potential as paleo-archive
CONSTANT source VS. trophics – suggests NO trophic structure shift –

Bulk shift caused by regime shift
Older coral values (back to AD 400..) suggest this is very recent shift – connected to recent regime shift (anthropogenic?)
A cautionary Aside:

**How good are these calibrations, and knowledge of “source” vs. “trophic”?**

*All published stuff has ALL (pretty much) based on one single study*
1) “limited information is available regarding basic pattern and $\Delta$ value w/ trophic transfer- and variability, despite the fact that they are essential factors in calculating trophic levels and understanding their precision. (Quite Correct.)

2) If these values vary across different samples, the amino acid method could lead to large errors in estimating trophic levels.

1): constant pattern algal pattern? If starting point different.. can’t calibrate accurately for a trophic transfer.

2) - trophics and source behavior depends on organism.?
Note: they did first two-trophic transfer test.  
(pretty cool)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Food</th>
<th>Δ¹⁵N (%o, relative to Air)</th>
<th>TL&lt;sub&gt;bulk&lt;/sub&gt;*</th>
<th>TL&lt;sub&gt;GLU/PHE&lt;/sub&gt;&lt;sup&gt;†&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bulk</td>
<td>Ala</td>
<td>Gly</td>
</tr>
<tr>
<td>This study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zooplankton</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brachionus plicatilis (#3)</em></td>
<td>Culture</td>
<td>Chlorella sp. (#1)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>2.0</td>
<td>0.8</td>
<td>-4.7</td>
</tr>
<tr>
<td><em>Brachionus plicatilis (#4)</em></td>
<td>Culture</td>
<td>Chlorella sp. (#2)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>-1.5</td>
<td>2.5</td>
<td>-4.7</td>
</tr>
<tr>
<td>Fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paralichthys olivaceous</td>
<td>Culture</td>
<td><em>B. plicatilis</em> (#4)</td>
<td>2.0</td>
<td>5.4</td>
<td>7.8</td>
</tr>
<tr>
<td>Sebastes schlegeli</td>
<td>Culture</td>
<td><em>B. plicatilis</em> (#3)</td>
<td>2.5</td>
<td>10.0</td>
<td>-1.0</td>
</tr>
</tbody>
</table>
Pattern data.

All normalized to phe = zero.

1) Looks pretty good. (*better hope Phe is constant.*)

2) Gly & Ser crazy.

1) Glu= solid?
Tropic transfer Summary:

**Table 4.** Summary of the mean original isotope difference between amino acid and phenylalanine (\(\beta_{\text{Phe}}\)) and \(^{15}\text{N}\)-enrichment factor along trophic level (\(\Delta\)) and their variability (1\(\sigma\)).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>(\beta_{\text{Phe}}) Average</th>
<th>(\beta_{\text{Phe}}) 1(\sigma)</th>
<th>(\Delta) Average</th>
<th>(\Delta) 1(\sigma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk</td>
<td>2.6</td>
<td>1.3</td>
<td>2.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.2</td>
<td>1.2</td>
<td>6.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>-2.3</td>
<td>3.4</td>
<td>3.7</td>
<td>3.9</td>
</tr>
<tr>
<td>Valine</td>
<td>4.6</td>
<td>1.2</td>
<td>5.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.3</td>
<td>1.6</td>
<td>4.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.9</td>
<td>0.8</td>
<td>4.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Proline</td>
<td>3.1</td>
<td>1.7</td>
<td>6.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Serine</td>
<td>-4.6</td>
<td>2.2</td>
<td>3.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>-2.0</td>
<td>0.6</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>3.4</td>
<td>0.9</td>
<td>8.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>—</td>
<td>—</td>
<td>0.4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Std deviations that are key – conclude that Glu and Phe are best!
Their Overall Big Conclusion:

“.. It just don’t change a thing..”

\[
TL_{\text{Glu/Phe}} = \left( \delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}} - 3.4 \right)/7.6 + 1
\]  \hspace{1cm} (7)

“Eq. 7 is very similar to the equation generally applied in previous studies …” using glutamic acid and phenylalanine with a $\beta$Glu/Phe value of 4\%, a $\Delta$Glu value of 7\%, and a $\Delta$Phe value of 0\% (Pakhomov et al. 2004; Schmidt et al. 2006; Hannides et al. 2009). Both equations provide similar results in estimating the trophic level. In the other words, this similarity proves the universality of the Eq. 7
BUT WAIT.
Looking more closely at data...

*Strong variation between replicates in many...wonder how good the data really is. Is n=2 or even n=4 enough?

How many individuals (species?) do you have to measure?

* Why such large variation for some AA between organisms types?
Glu and Phe (and met) have most consistent behavior of the ones they measured.

Gly and Ser have LEAST consistent behavior—varies dramatically with type of consumer!

* Note both supposedly are “source”—but both seem highly organism dependent)

Eg: GLY is only “source” in plankton.. NOT in gastropods or fish.. Ser is never “source” in this data..
A basic Q: Source AAs?

Take step back:
You look at this picture- does the “two group” thing holds up?
IE, do you see two groups here?

It looks to me that like once you leave plankton- there are only TWO “source” AA in this data- and really maybe only one..

“More work is needed”
I ENDED HERE-

3 FEB-2010, Paul’s class

however if you are interested CSI-AA uses in detrital, organic matter, continue on..
3) New indicators of processing AND heterotrophy in detrital organic pools?
First a Basic Question: is this information preserved in DETRITAL pools? (PON, DON)

Amino acid nitrogen isotopic fractionation patterns as indicators of heterotrophy in plankton, particulate, and dissolved organic matter

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d Carnegie Geophysical Laboratory, 5251 Broad Branch Rd. N.W., Washington, DC 20015, USA
Mixed Zooplankton vs. Diatoms: works great

△ ~ 7‰

Trophic source
Sinking Ocean Particles (POM), 1000 & 3600m: **YES!**
Trophic Position of exported POM?
Predictions match very well with independent data

BUT: are smaller variations meaningful?

- 2N- tow => 105m
- export ~ +0.5

⇒ Independent data suggests YES!

- Zang et al., 1995: 2x grazing rates, N vs S
- Hernes et al., 1996; Lee et al., 2000 (carbos, lipids say incr. degradation in N)
BUT what about Degradation? (ie, the microbial wildcard)

*In detrital OM, problem is patterns may not stay same way for long.*
Rather understanding cycling of ON boils down to: what is effect of bacterial activity?
Extremely uniform “enriching” $\delta^{15}N$-AA pattern in N an opportunity?

_T. suecica #1_

McClelland and Montoya, 2002
Some Detrital POM: much more scatter
Hypothesis: Scatter due to Bacterial Resynthesis
If True:

Measure of increased “scatter”
relative to algal or zooplankton pattern

= prokaryotic heterotrophic resynthesis?
Proposal:

We Can define a parameter - $\Sigma V$ (average deviation in E-AA group)

= Measure of total resynthesis?
$\Sigma V$ vs. Sample Type

- **Phytoplankton**
  - *T. Suecica*
  - *Rhizosolenia* sp.
- **Zooplankton**
  - *B. Plicatilis*
  - Mixed Tow, 2N
- **Surface Traps**
  - Equatorial Traps, Strong El-Niño
    - 105m, 2N
    - 105m, 1S
- **Deep Traps**
  - Year-long Integrations N and S of Equator
    - 1000m, 5N
    - 3600m, 5S

Offset Not observed In $\Delta Tr$

But follows Mol% Gly and DI index trends!

(Lee et al, 2000)
ΣV vs. ΔTr:
A two dimensional “heterotrophic space”?
Implication from Eq. Pacific Data:

Eukaryotic and Prokaryotic heterotrophy very decoupled in space (and time)?
δ\textsuperscript{15}N-AA summary

“A new set of tools for N source and transformation”

1) δ\textsuperscript{15}N at base of food web (unaltered by trophic transfer?)

2) # of trophic transfers (Δ Tr = Internally normalized index)

3) index for total AA resynthesis? (Σ V)

→ 2-D “heterotrophic space” relative measures prokaryotic vs. eukaryotic heterotrophy?

4) could also be Information on Source??
4) 15N-AA Source indicators?

Are there Diagnostic evolutionary pathway differences for N, as there apparently are for carbon?

Larsen et al, 2009- carbon data showing tracer potential
Trace: Prokaryotic vs. Eukaryotic N inputs?
Small Phytoplankton and Carbon Export from the Surface Ocean

Tammi L. Richardson and George A. Jackson

2007

Autotrophic Prokaryotes >75% Zooplankton ~90% Fecal Pellets Autotrophic Eukaryotes
General pattern is very similar exemplifying a common autotrophic signature - BUT..

*One key difference* is seen in Glx isotopic value.
Differences in Glx could be related to differences in Glutamine Synthetase

Glutamine synthetase is transcribed from 3 different gene families

- GS-I → archaea & bacteria
- GSII → eukaryotes
- GSIII → mostly prokaryotic

Gill & Eisenberg (2001)

Llorca et al. (2006)
Recall AA fall into two groupings relative to Glx

- Fundamental split in N metabolism between the 2 groups
- Glx signal may be retained in the “non-fractionating” AA
Fractionation coefficients for several AA’s also show clear differences between two domains.

Are these differences related to actual differences in biochemistry?
PCA of all AA yields separation of domains

![PCA plot]

- **Eukaryotes**: △
- **Prokaryotes**: ✧
PCA of AA normalized to $\delta^{15}$N of Glx yields separation in both groupings

**“Non-Fractionating” AA**

ANOVA = 0.0157

**“Fractionating” AA**

ANOVA = 0.0186

△ Eukaryotes

◆ Prokaryotes
DFA output is not as good based on the two classification -

"Non-Fractionating" AA
Pillai's Trace p value = 0.3645

"Fractionating" AA
Pillai's Trace p value = 0.2355

△ Eukaryotes  ◆ Prokaryotes
However, the “selected” AA model assigns samples into domains significantly.

Selected AA’s: Glx, Asx, Ala, Val, Pro, Gly, Leu, Phe

Non-fractionating grp

Diff. Coef’s ?

Pillai’s Trace p value = 0.0317

"Selected" AA

Pillai’s Trace p value = 0.0317

△ Eukaryotes  ● Prokaryotes
Summary of Multivariate Analyses

- PCA was able to separate domains!

- DFA: stepwise selection model correctly predicted the domains of each culture
  - “chose” AA previous observations implied had differences
III: A few examples of applications and spin-offs

(and even a few things I never thought would be going on in my lab in a zillion years..)
AA isotopic patterns in seal blood a tracer of central metabolism? (nutritional state?)
HOLY TOLEDO!
• Some Challenges:

* How Constant (i.e., predictable) are shifts of Trophic AA in different ecosystems? (land vs. sea?)

* How “stable” are selected source AA’s really? How far up food chains do they maintain their signatures?

* How does stress or state change things—of an organism? Of a population?
There’s way more..
Really interesting stuff about
DOM, etc..

But another day.