Molecular methods for monitoring in IRZ+PRZ

Greg Rouse
Scripps Institution of Oceanography

WORKSHOP ON
THE DESIGN OF "IMPACT REFERENCE ZONES" AND "PRESERVATION REFERENCE ZONES" IN DEEP-SEA MINING CONTRACT AREAS

Sept 27-29 2017

OUTLINE

Collecting for DNA (& Morphology)

Curation Pipeline for DNA (& Morphology)

DNA, caution & recommendation

Data consistency & sharing is important

Rapidly developing DNA sequencing technology

Collecting for DNA (and Morphology)

- Requirements will vary according to location.
- For the deep sea:
- Keep samples **cold**!
- Process samples with **cold** seawater!
- Process samples gently!
- Get samples isolated and into clean seawater and into a **cold** room as soon as possible.

General Advice on Specimens

RELAXATION

• PURPOSE - to anaesthetize specimen so it is unable to respond or contract when placed in fixative.

• Important for humanitarian reasons

• Identification often hampered or impossible if fixed in a contracted form

• prevent autotomy (drop legs, claws, foot, etc.) - if dropped straight into fixative

Relaxants

$MgCl_2$

Freshwater at 7-7.5% weight, isotonic with seawater (add 70-75 g to 1 liter freshwater). Note $MgCl_2$ crystals are highly hydrophilic, if wet you will need to mix a generously greater amount. Exact percentage is not critical. Works by competing with Ca in muscles and nerves, making animals unable to contract. A 50:50 mixture of isotonic $MgCl_2$ solution: sea water is a good general mix to use; $MgCl_2$ solution should be gradually added to seawater for especially sensitive animals. Be patent!

Clove oil = eugenol

Relaxes most crustaceans rapidly. Prepare a saturated solution in sea water, and add to bowl containing animals.

Chloretone = chlorobutanol

Chloretone is not readily miscible in water, so it is prepared in a saturated ethanol solution (a large amount o the chloretone can be dissolved in a volume of alcohol). A couple of drops in a bowl or a pipette full to a bucket works well on echinoderms, including large holothurians.

Menthol

Add to dish with animals by either sprinkling crushed crystals on top or adding drops of concentrated mentho solution prepared in ethanol. Menthol works especially well for cnidarians and ascidians.

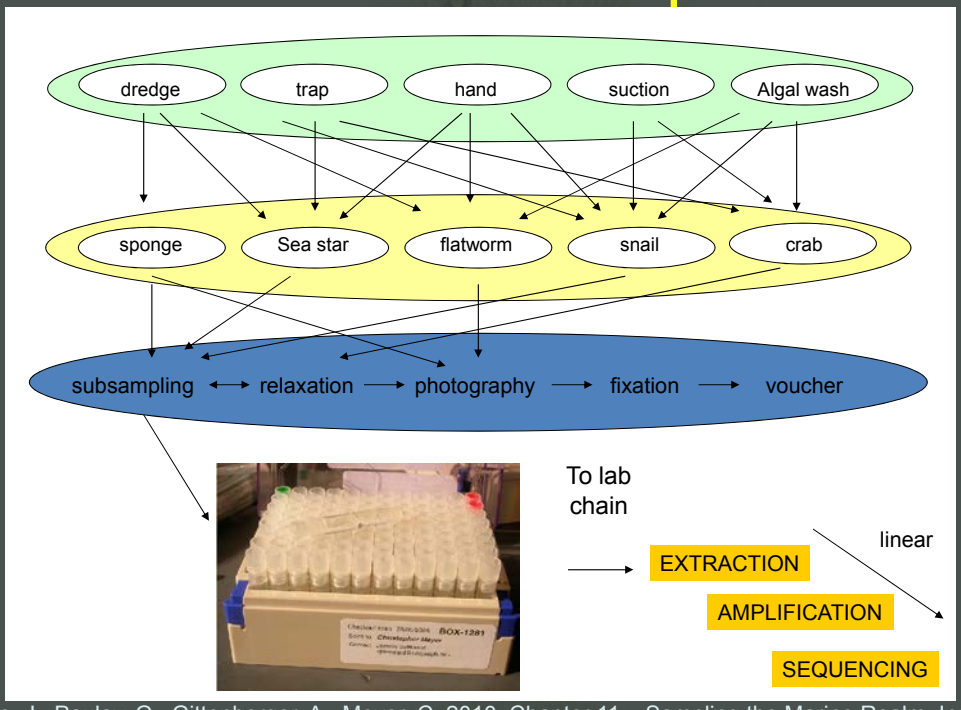
General Advice on Fixing Specimens

Specimen Handling

	TAXON	Tissue subsample	relaxation	photography	fixative	preservative	notes
	Porifera	avoid internal and epibionts	none	In situ	Ample 80-95% ETOH, transfer to clean alcohol in 1 week+	ETOH	Record internal + external color, texture, surface feel, odor , mucous production, etc.
	Cnidaria	Tentacle best	Menthol, MgCl	Live animal, in situ best	formalin	formalin	See specific instructions for soft corals, gorgos, black corals, anemones, scelractinians, jellies
	Flatworms	Small snippet	none	Live animal essential	Crawl on paper, place gently onto frozen formalin	ETOH	Must take care in fixing - have frozen formalin ready
	Annelida	Half mid-body, not head or tail	MgCl	Photos useful	formalin	ETOH	Try to avoid gut contents in subsampling
	Crustacea	leg	Freeze generally, MgCl or clove oil as well	Important as color can be lost in preservation, freshly killed, with legs spread	80-95% ETOH	ETOH	Avoid quick fixation - will drop appendages, living photo of shrimp or translucent, much better
	Mollusks*	foot	MgCl propylene phenoxital, snap boiling, etc - varies	Not critical, important if distinctive characteristics (coloration, mantle, etc.)	Formalin or Bouin's	ETOH	Shells available, adequate relaxed soft parts are lacking, *cephalopods and opisthobranchs different
	Holothuroids	Inner longitudinal body wall muscle best, gonad OK	chloretone	Field/live photos useful	Inject and fix with 80-95% ETOH	ETOH	Dilute down to 70-80% with body fluids
	Ascidians		menthol	In situ	formalin	formalin	Prepare menthol in alcohol

Templado, J., Paulay, G., Gittenberger, A., Meyer, C. 2010. Chapter 11 – Sampling the Marine Realm. In: Eymann, J.; Degreef, J.; Häuser, C.; Monje, J.C.; Samyn, Y and VandenSpiegel, D (eds). Manual on field recording techniques and protocols for All Taxa Biodiversity Inventories and Monitoring. Abc Taxa, Vol. 8 (Part 1): 273-311.

General Advice on Specimens



Templado, J., Paulay, G., Gittenberger, A., Meyer, C. 2010. Chapter 11 – Sampling the Marine Realm. In: Eymann, J.; Degreef, J.; Häuser, C.; Monje, J.C.; Samyn, Y and VandenSpiegel, D (eds). Manual on field recording techniques and protocols for All Taxa Biodiversity Inventories and Monitoring. Abc Taxa, Vol. 8 (Part 1): 273-311.

Deep Sea Samples, 3000 m. Multicore Suspension Decantation: 250 µm sieve

DNA/RNA

Photograph specimen, keep a voucher!

1. For DNA, animals can be dead but not for too long. Try and keep animals alive. Relax specimen
2. Plunge freeze in liquid N if possible or use -80 freezer.
3. If not, use 95% ethanol (DNA only).
4. Rinse and change ethanol within a week. Change again if there is pigment
5. Store at -20C or -80C when possible

Also see detailed pipeline re CCZ

Glover et al. 2016. An End-to-End DNA Taxonomy Methodology for Benthic Biodiversity Survey in the Clarion-Clipperton Zone, Central Pacific Abyss J. Mar. Sci. Eng. 4, 2 doi:10.3390/jmse

DNA Sequencing: caveats

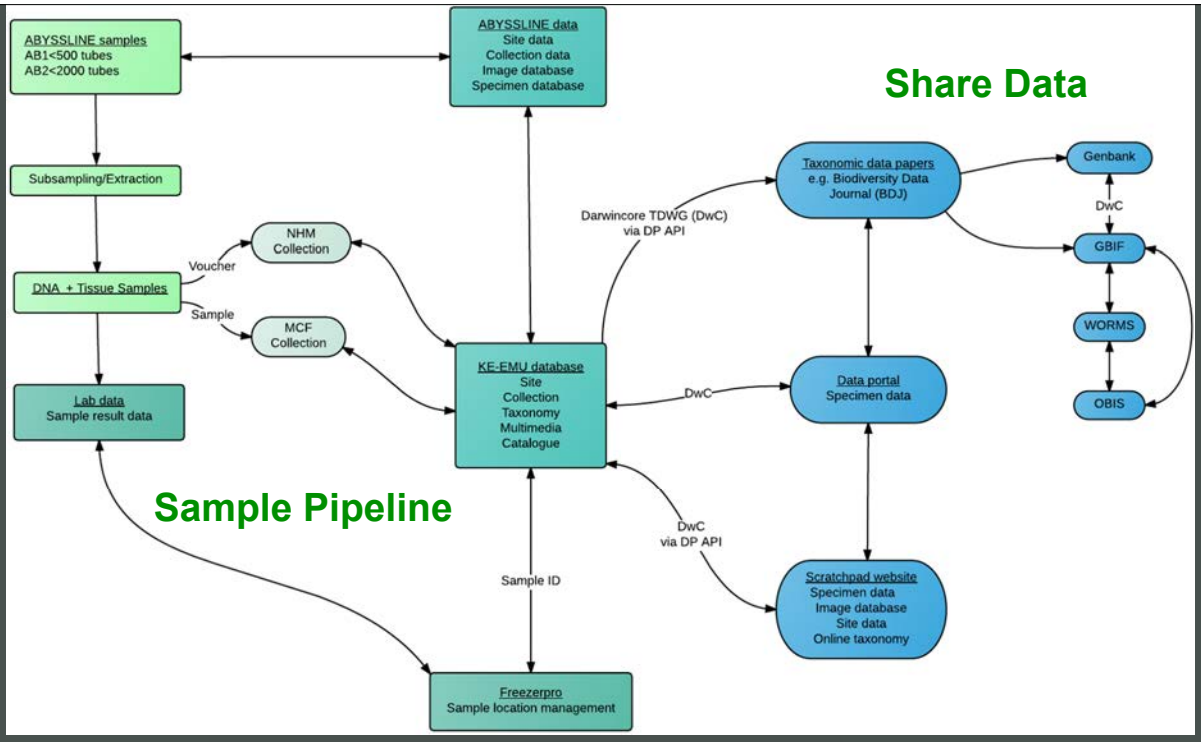
- Sequencing technology and methods are developing very quickly
- Prescribing methods and requirements unwise for the long term
- BUT, **DNA barcoding** (in various modes) has been around since 1994, has critical mass, and is largely suitable for documenting biodiversity

Folmer, O., Black, M., Hoeh, W. R., Lutz, R. A., & Vrijenhoek, R. C. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*(5), 294 -299.

DNA Sequencing, recommendation

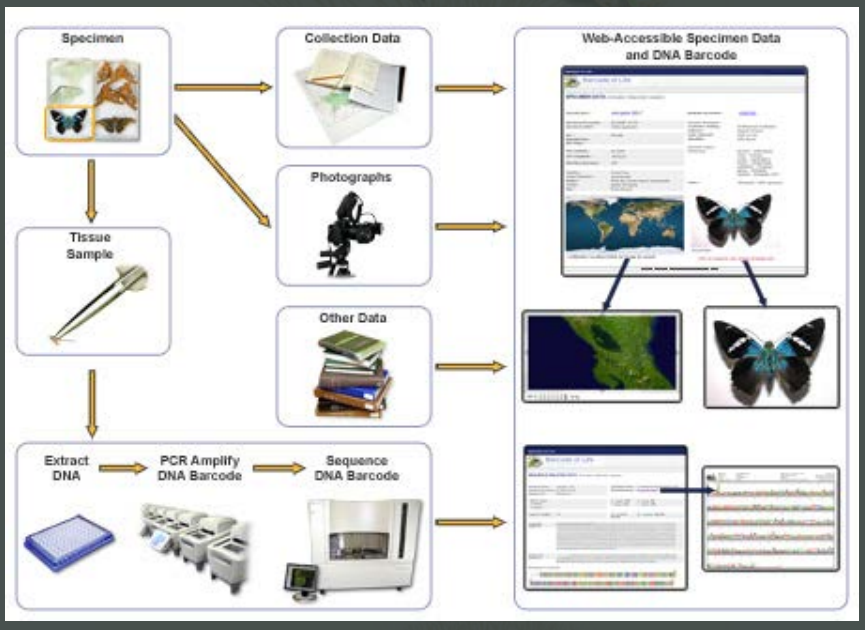
- **Minimal** sequencing (= DNA '**barcoding**') will vary by taxonomic group but is now well established

- Animals: Mitochondrial, *COI*; supplemented by *16S rDNA* for groups with slowly-evolving COI (Cnidaria, Porifera). Nuclear, *18S rDNA* variable region is favored for nematodes.
- Fungi: Nuclear, internal transcribed spacer (*ITS*)
- Foraminifera, Diatoms etc: Nuclear, *18S rDNA* variable region
- Bacteria/Archaea: *16s rDNA* libraries



Glover et al. 2016. An End-to-End DNA Taxonomy Methodology for Benthic Biodiversity Survey in the Clarion-Clipperton Zone, Central Pacific Abyss J. Mar. Sci. Eng. 4, 2 doi:10.3390/jmse

Partial *COI, 16S, ITS* etc. each cost ~US$5-6 per specimen + labor, 96-well plate extraction & sequencing



DNA barcoding pipeline

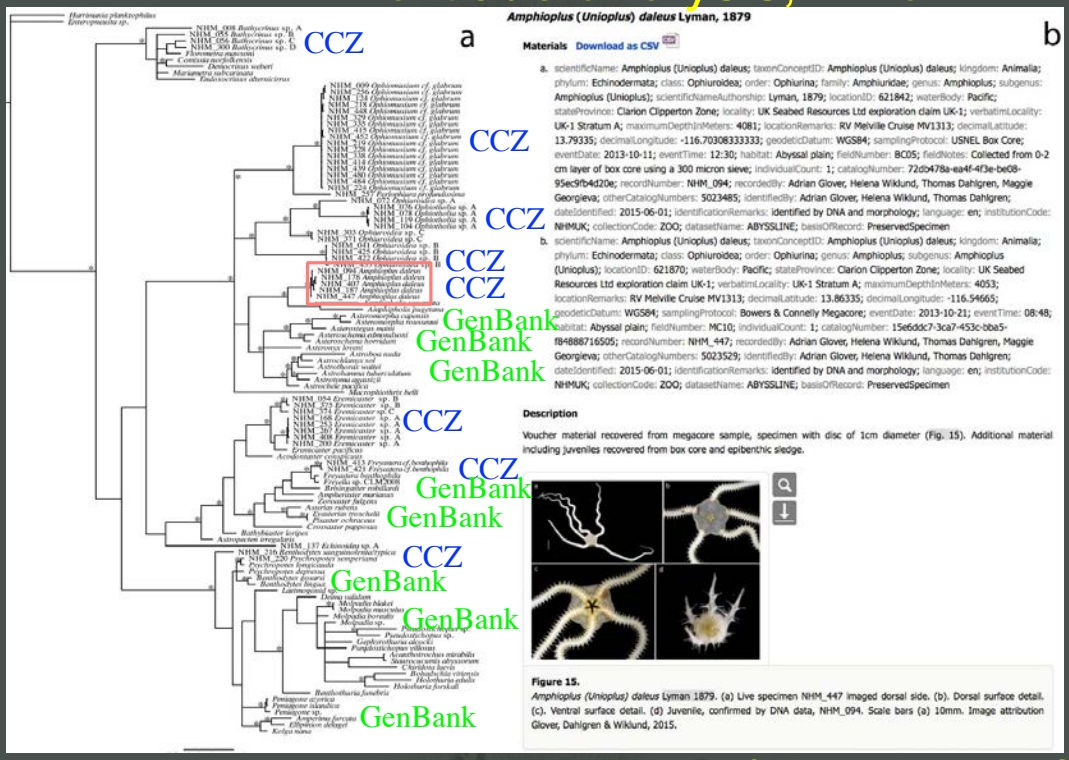
http://www.ibol.org/phase1/about-us/what-is-dna-barcoding/

DNA Barcoding doesn't alway work easily……

- High quality material from the beginning will make a huge difference
- Small body size = problem vouchering; enough DNA.
- Also, empirical studies show 'universal barcode' primers (e.g., classic COI= 'Folmer') fail in some groups when covering a broad taxonomic range.
- e.g. 44% success in more than 2000 initial amplifications in the Moorea Biocode Project

Geller J, Meyer C, Parker M, Hawk H. 2013, Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. Mol. Ecol. Resour. 13, 851 – 861. (doi:10.1111/1755-0998. 12138) .

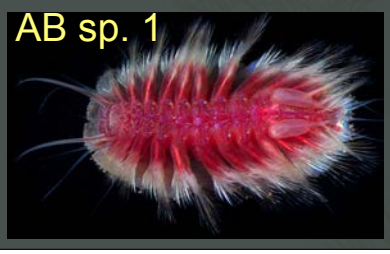
DNA Barcode analysis, initial



Glover et al. 2016. An End-to-End DNA Taxonomy Methodology for Benthic Biodiversity Survey in the Clarion-Clipperton Zone, Central Pacific Abyss J. Mar. Sci. Eng. 4, 2 doi:10.3390/jmse

Data consistency & sharing is important

Mining area A has sample species **Xy sp.1**; another has **AB sp. 1**
Calling different things the same, or calling the same thing different?
Clearly has implications for PRZ and IRZ
Being able to check DNA Barcodes may show they are the same thing.
Or different

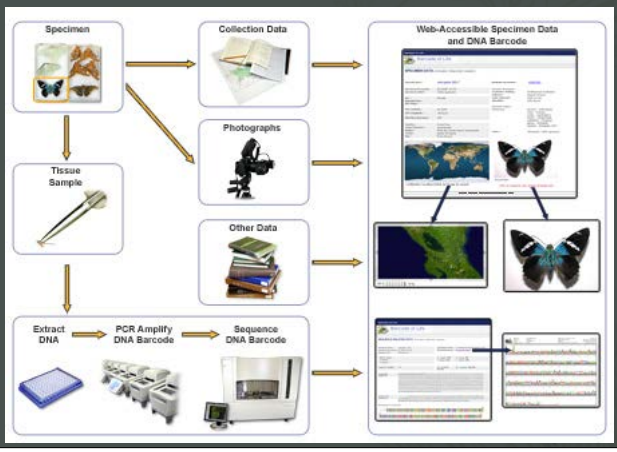


Data Sharing, recommendation

Coordinate DNA and other collection data among different claimants
Simplest way is to use available portals, GenBank/BOLD, + ISA itself

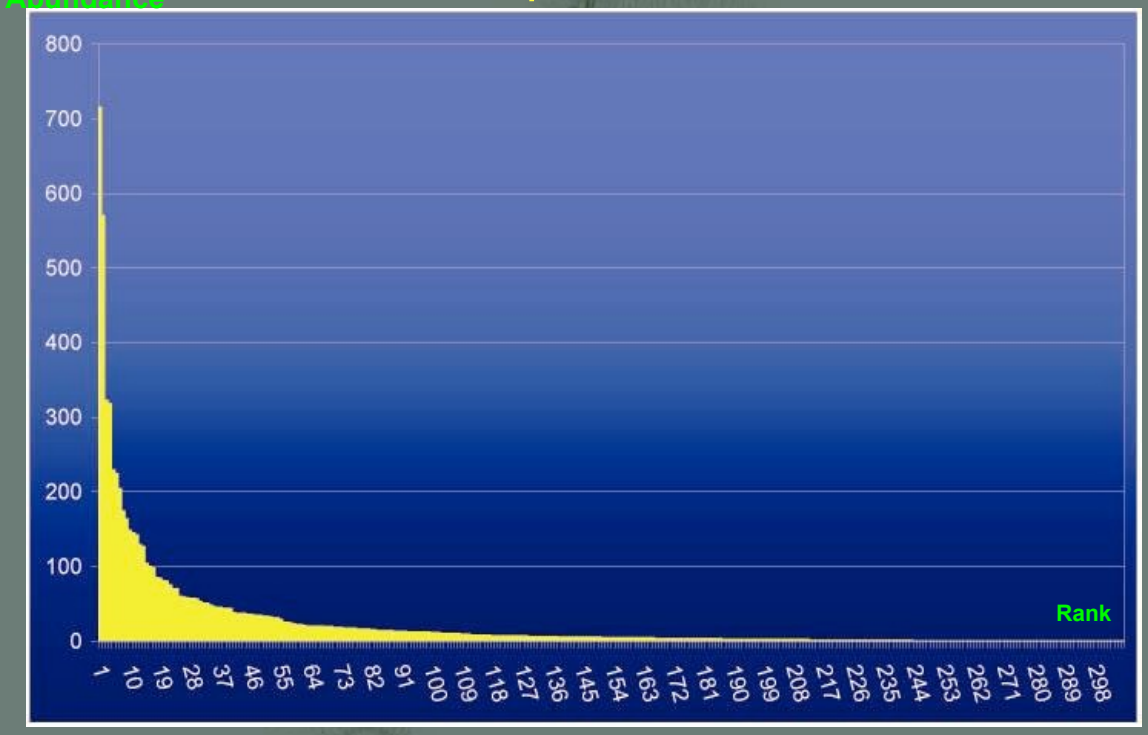
Larger, broader and more complete curated DNA database for a region allows better decisions to be made

DNA Barcoding & vouchering pipeline is laborious & time consuming



But it can be a 'one off' (collaborative) effort for a region that will accumulate and have long-term benefits in terms of monitoring

Most Marine Species Are Rare



Turridae (gastropod snails) from Lifou (New Caledonia)

Philippe **Bouchet**

Ongoing monitoring after establishing the baseline continue as before

Or….

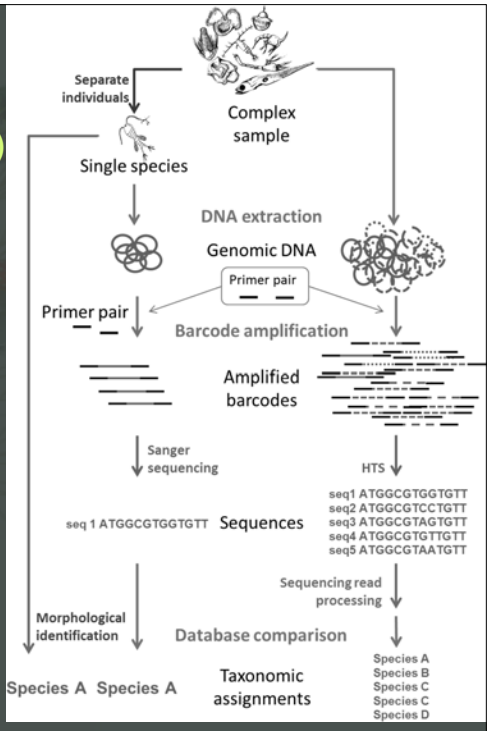
DNA Metabarcoding

via High-throughput DNA sequencing (HTS)

or Next-Generation Sequencing (NGS)

Metabarcoding utilises the same principle as classical DNA barcoding, but with much higher throughput, allowing simultaneous processing of hundreds of specimens in a single analysis.

Metabarcoding primer ‘cocktails’ designed to amplify the full COI barcoding region available for marine animals

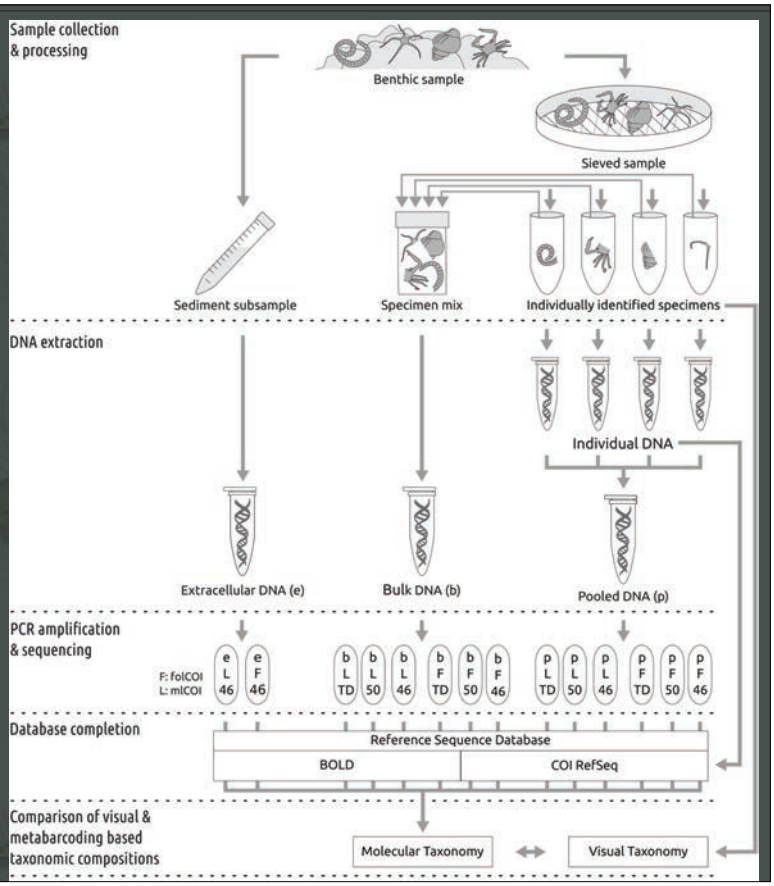


Bucklin et al 2016. Metabarcoding of marine zooplankton: prospects, progress and pitfalls
J Plankton Res. 38: 393-400. doi:10.1093/plankt/fbw023

Metabarcoding

Illumina© platforms provide **millions** of reads per run, hundreds of specimens may be sequenced simultaneously.
Hundreds of COI reads per specimen (=QC) at less than $1 each

Aylagas, E., Borja, A., & Rodríguez-Ezpeleta, N. (2014). Environmental status assessment using DNA metabarcoding: towards a genetics based Marine Biotic Index (gAMBI). PLoS ONE 9:e90529.



Problems with metabarcoding

In standard DNA barcoding, it is possible to optimize protocols to get data from specimens that initially fail to amplify.

Metabarcoding of a DNA mixture can have failed amplifications of particular taxa.

Masked by the recovery of sequences from other species in the sample. You wont even know it failed……

Also low recovery of rare organisms in COI metabarcoding

Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., and Taberlet, P. (2014). DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. Biol. Lett. 10:20140562.

Matthieu, L. and Knowlton, N.. 2017. Random sampling causes the low reproducibility of rare eukaryotic OTUs in Illumina COI metabarcoding. PeerJ, 5 doi:10.7717/peerj.3006

NGS metabarcoding for quantitative assessment?

Ecological function of species and community structure is important.
Occurrence AND abundance in communities is required.
Will NGS allow for abundance estimation?

Currently…….. NO

Elbrecht, V., and Leese, F. (2015). Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass–sequence relationships with an innovative metabarcoding protocol. PLoS ONE 10:e0130324. doi: 10.1371/journal.pone.0130324

Matthieu, L. and Knowlton, N.. 2017. Random sampling causes the low reproducibility of rare eukaryotic OTUs in Illumina COI metabarcoding. PeerJ, 5 doi:10.7717/peerj.3006

eDNA

Environmental DNA or eDNA = DNA from environmental samples (seawater, sediment) rather than directly sampled from an organism

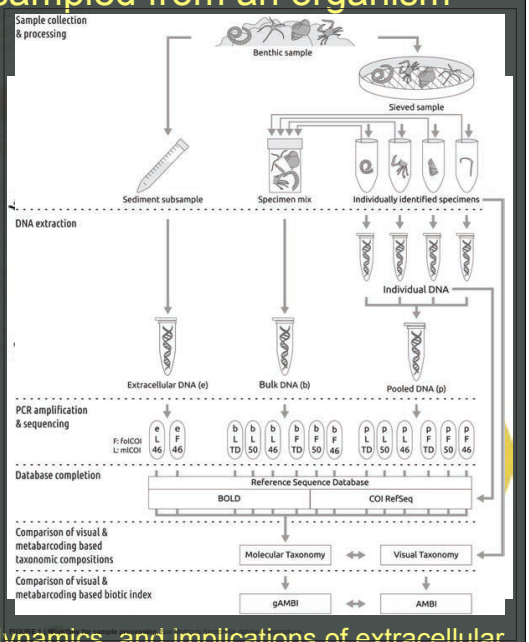
eDNA may account for the largest fraction of total DNA in sediments

Comprises genetic material released in situ from sediment communities, as well as DNA of pelagic origin deposited on the seafloor.

May allow detection of megafauna in small samples, facilitating sampling strategies and cost-effective biomonitoring.

Age of the eDNA uncertain.

Undermines the direct link between the overall DNA pool and the local, currently living community



Torti, A., Lever, M. A., and Jørgensen, B. B. (2015). Origin, dynamics, and implications of extracellular DNA pools in marine sediments. Mar. Genomics 24, 185–196. doi: 10.1016/j.margen.2015.08.007

Conclusions

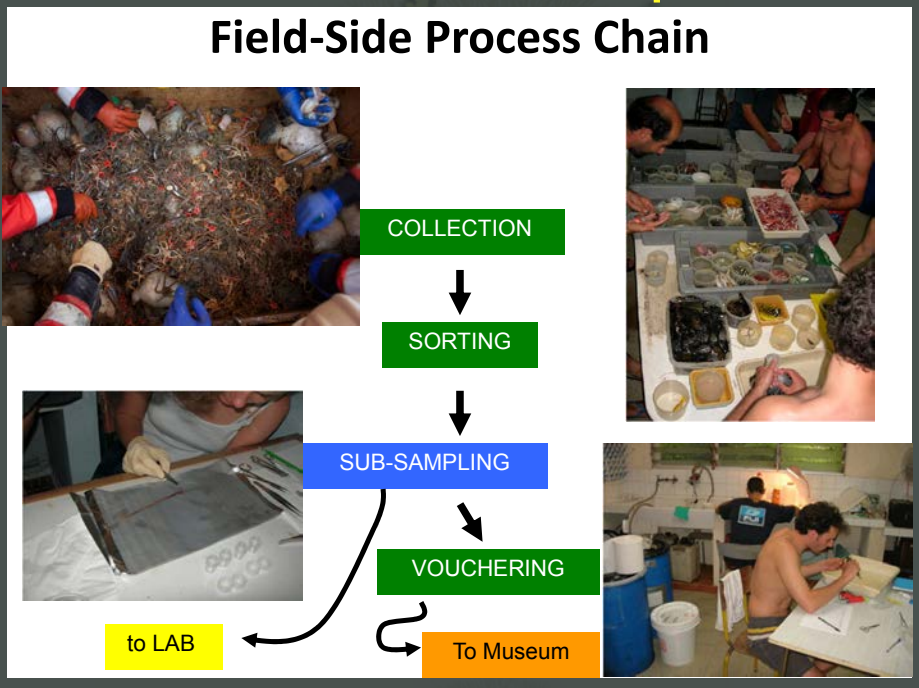
Sample processing is important, has major downstream consequences

DNA Sequencing: identifies, quick, standardized, universal, cheap!
DNA Sequencing: technology rapidly evolving
Recommendation = curated DNA Barcoding

Data sharing is important, reduces redundancy. Has many broad benefits for contractors, monitoring requirements and science
Recommendation = Coordinate DNA and other collection data among different claimants using available portals, GenBank/BOLD, + ISA itself

Rapidly developing new DNA sequencing technology, HTS/NGS, means once the baseline work is done then ongoing monitoring will be cheaper and less laborious.

General Advice on Specimens



Templado, J., Paulay, G., Gittenberger, A., Meyer, C. 2010. Chapter 11 – Sampling the Marine Realm. In: Eymann, J.; Degreef, J.; Häuser, C.; Monje, J.C.; Samyn, Y and VandenSpiegel, D (eds). Manual on field recording techniques and protocols for All Taxa Biodiversity Inventories and Monitoring. Abc Taxa, Vol. 8 (Part 1): 273-311.