MOLECULAR IDENTIFICATION AND ENUMERATION OF INVERTEBRATE LARVAE POTENTIALLY ENTRAINED BY ONCE-THROUGH-COOLING

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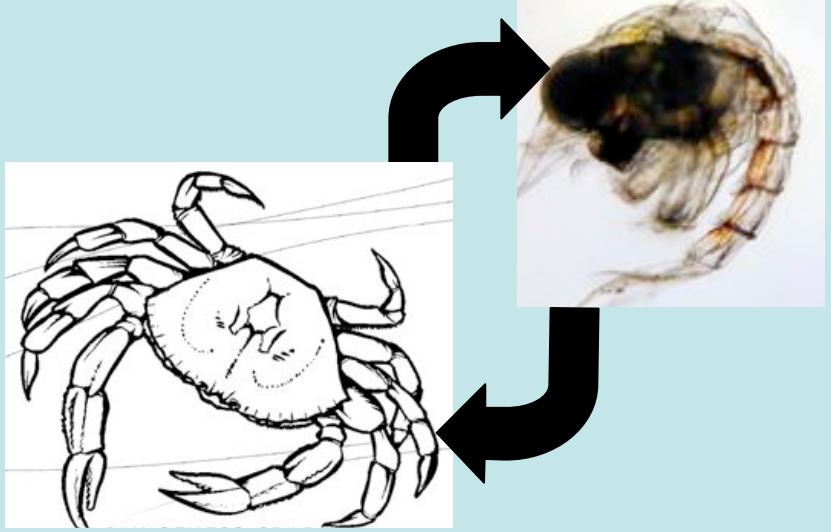
Goals:

Characterization of larval assemblages by DNA barcoding.
Develop and assess protocols for enumeration of larvae by qPCR.

How are invertebrate populations affected by mortality of larvae in once-through cooling systems?

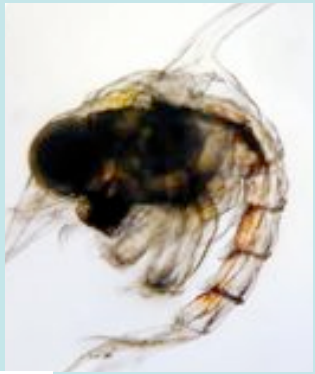
Larvae are most directly impacted, but most larvae cannot be readily identified.



Problem to solve:

- Identification of relevant life history stages.

Even if larvae were identifiable, quantitation is slow and laborious



Problem to solve:

- Faster ways to quantify populations

Molecular methods have potential to solve these problems.

1. Detection of species-specific genetic markers.
2. Quantitative analysis of abundance of genetic markers.

We attempted this using a model larva in plankton samples drawn from Elkhorn Slough

Goal 1. DNA Barcoding

A. CREATING BARCODE DATABASE

Identified organism

Photography, vouchering, etc…

DNA extraction, PCR, DNA sequencing

Barcode Database

(eg, Consortium for the Barcode of Life)

B. USING BARCODE DATABASE

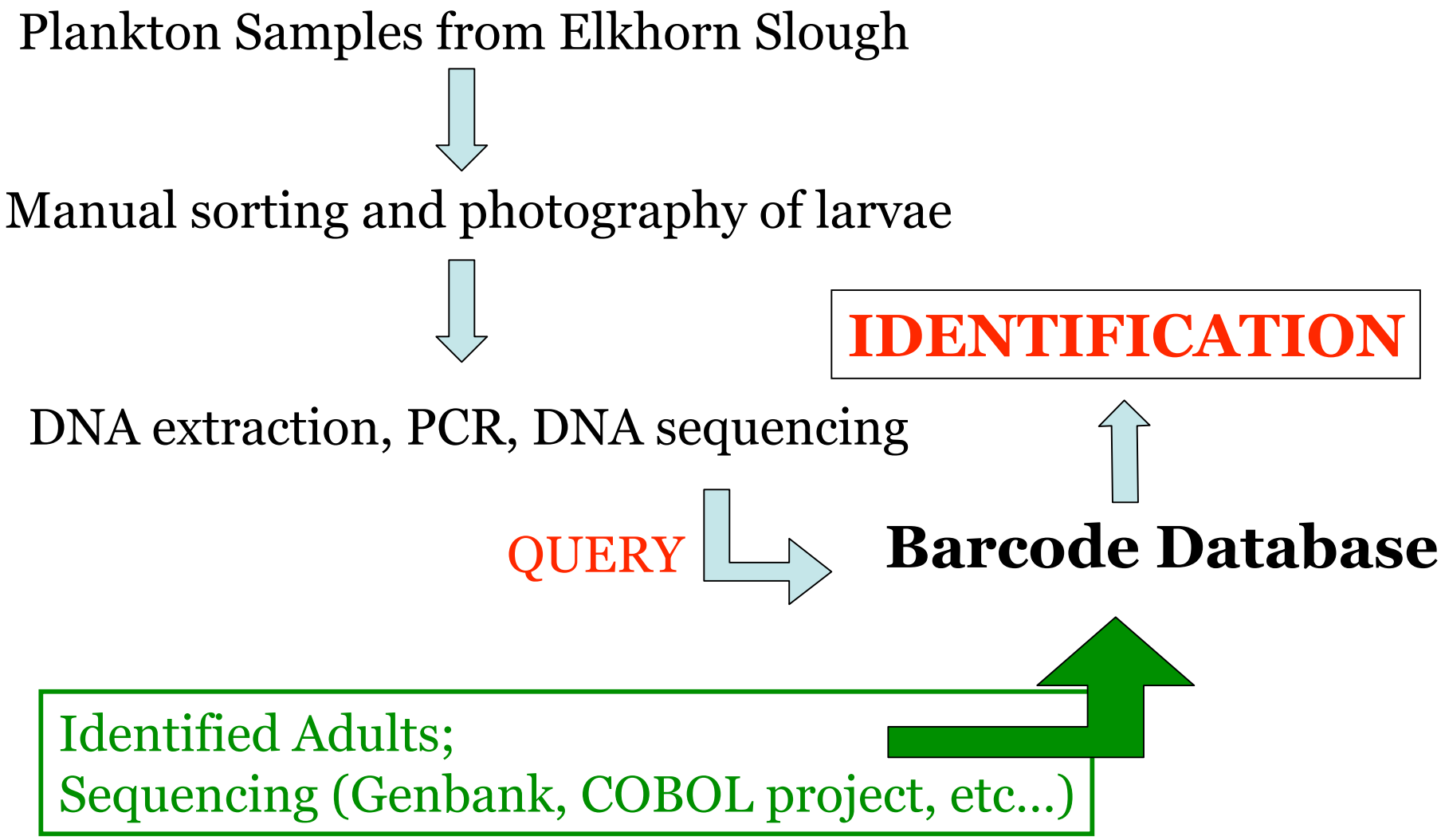
Unidentified organism

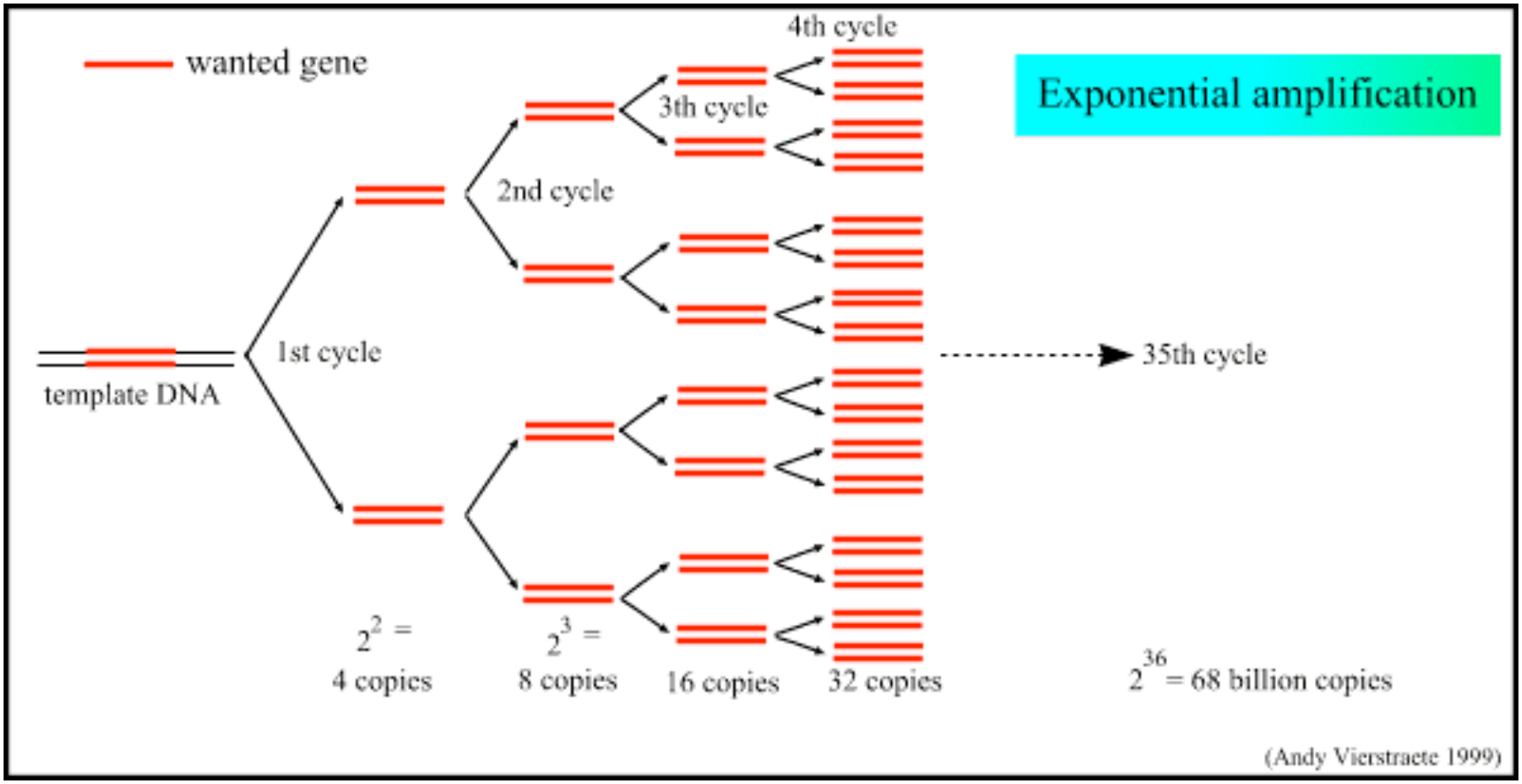
DNA extraction, PCR, DNA sequencing

QUERY

IDENTIFICATION

Larval DNA Identification with DNA Barcoding

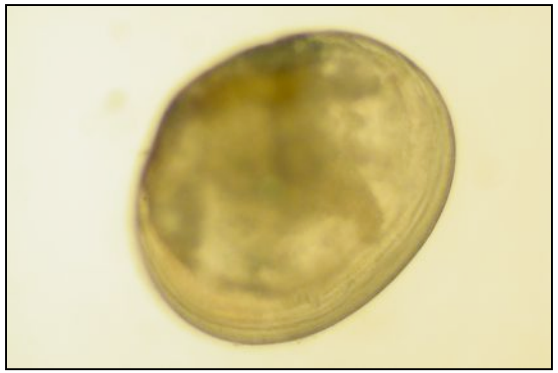




For studies of metazoa, the mitochondrial gene *Cytochrome c oxidase subunit I* (COI) is the standard for DNA barcoding.

Building a barcode database for Elkhorn Slough

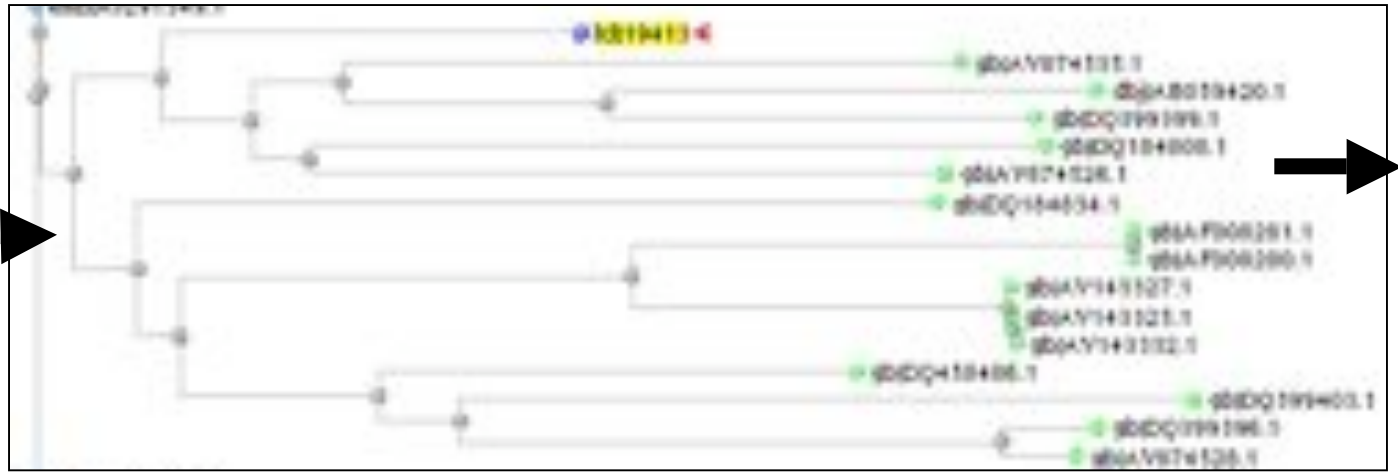
DNA was extracted, PCR and sequencing done...



Sequence queried against Genbank

```
>6590_bivalve_E4_4A_070129_COI_StopAACTTCAGGGGTGACCAAAAA
ATCAAAACAAATGAACAAAAAGAAGGGGATCACCTAAACCTAAAGGGTC
AAAAAAAAGTTGTATTTAAATTACGATCTGTTAACAACATAGTAAGACCA
GCAGCTAAAACAGGTATAGCACACAATAGTAAAAAACCAGTAATACAAA
CACACCATACAAATATAGTAGTTCGAGATATAGTTAAAATATCTGGACGT
CTAGAAAACCCAGTTCCAGCAAAATTAATACCAGACATAATAGAAGATG
CTCCTCCAACATGTAAAGAAAAAATTAAATAGTCAACAGAAACCCCCGA
ATGAAACTTTCATCTCGAAAGAGGGGGATAAATAGTTCAACCAGTCCCA
GCTCCAGCATCAACATAAGCAGACCCTAAAAATAATATCATAGAAACTA
AAACTAATCAGAAACTCAAATTATTTACCCGAGGATAAGCTATATCCGGT
GCAGTTAATATCAAAGGAACTAATCAATTACCAAAACCCCCAATTATCAT
TGGCATTACTAGGAAAAAAATTATAACCAAGCCATGAGAAGTAACAATT
AAATTATATAAATGAGCATCATCAAGAATAGTGCCAGGCATAGATAATTC
CATTCGAATTAAAATTCTAAACCTAGTACCCATTAAACCAGATCAAAAAG
CAAAAATCAGGTAAAGAGTGCCAATATCTTTATGATTTGTTGACCACCCT
GAAGTTTAAGCCTGGCTATGGTGTAGGGCCGAATTTTTCAGCCAGGGT
TCGGTTTAA
```

Accession	Species/Locus	E Value
AB059420.1	Meretrix lamarckii mitochondrial COI gene for cytochrome c oxidase subunit 1,	7e-128
DQ399399.1	Meretrix meretrix haplotype WF1 cytochrome c oxidase subunit I (COI) gene,	3e-126
DQ184808.1	Costacallista impar voucher AMNH 311623 cytochrome oxidase subunit I (cox1)	4e-125
AY874526.1	Cyclina sinensis cytochrome oxidase subunit I (COI) gene, partial cds;	3e-116
AY874535.1	Saxidomus purpuratus cytochrome oxidase subunit I (COI) gene, partial cds;	3e-97



Likely a venerid with no known sequence.

12 biweekly plankton samples; hand sorted for unique larvae.
911 individual larvae photographed and DNA extracted.
498 PCR amplifications (6 of 11 96-well plates of extractions).
251 successful PCR (51%) [success rate did not vary among taxa]
140 successful DNA sequences

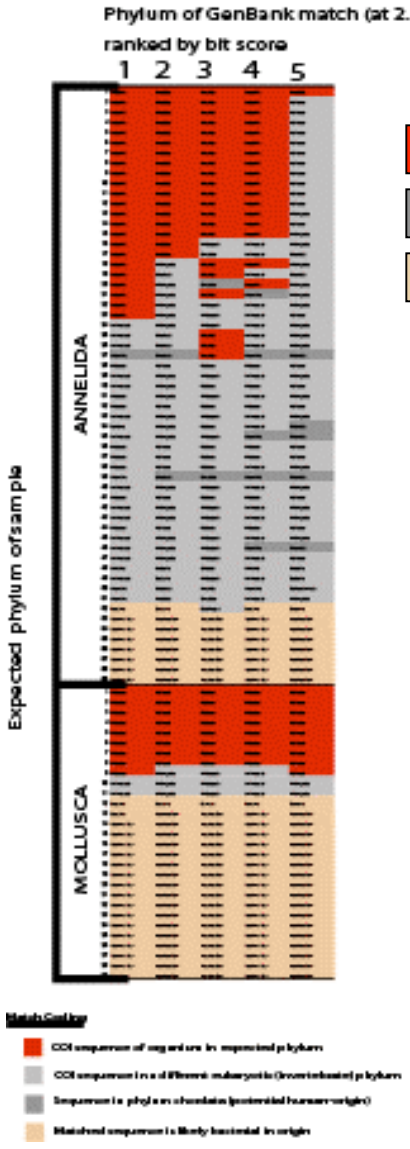
Overall, not satisfactory success rate.

Some problems seem specific to this project.
•High throughput DNA extraction procedures inadequate.
•Sequencing facility; quality problems.

These are solvable and not of fundamental concern.

All data now in accessible database.
www.mlml.calstate.edu/inverts

Database searches not yet effective



Correct phylum

Incorrect phylum

Bacteria

Hitting the incorrect *phylum* sounds bad, but is not a big problem as barcode database improves.

Bacterial contamination maybe site specific; Can be solved by improved primer design, though "universal" primers may not be obtainable.

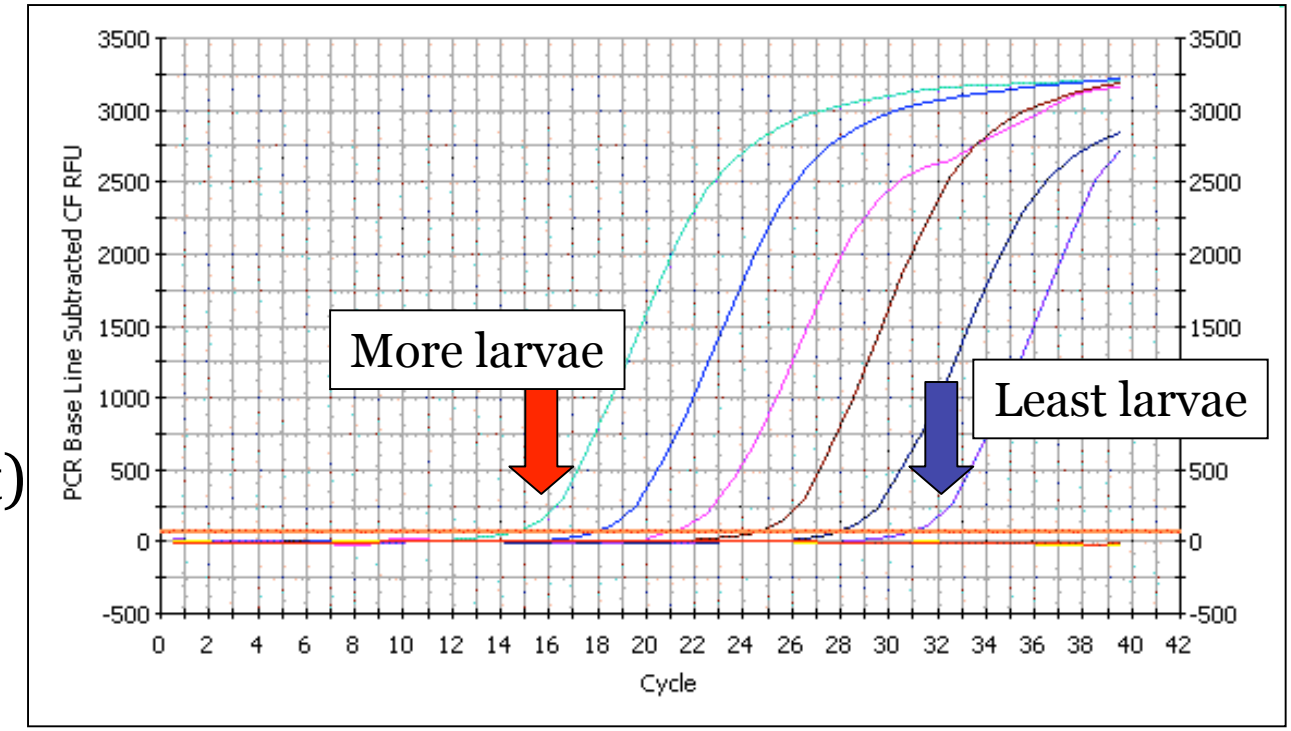
We also experienced difficulties getting good photographic records of live, moving larvae

Biggest problem: labor intensiveness. ID of larvae by DNA barcodes will have its place, but not for quantitative ecology

Goal 2: Quantitation of larvae by qPCR

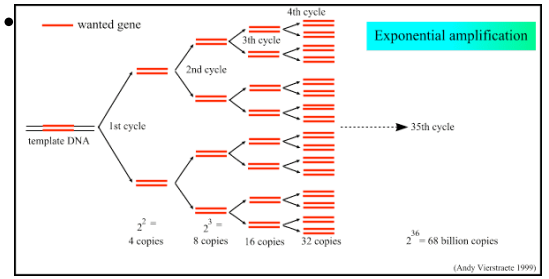
(also known as Real-Time PCR)

Fluorescence reports DNA (PCR product) accumulation

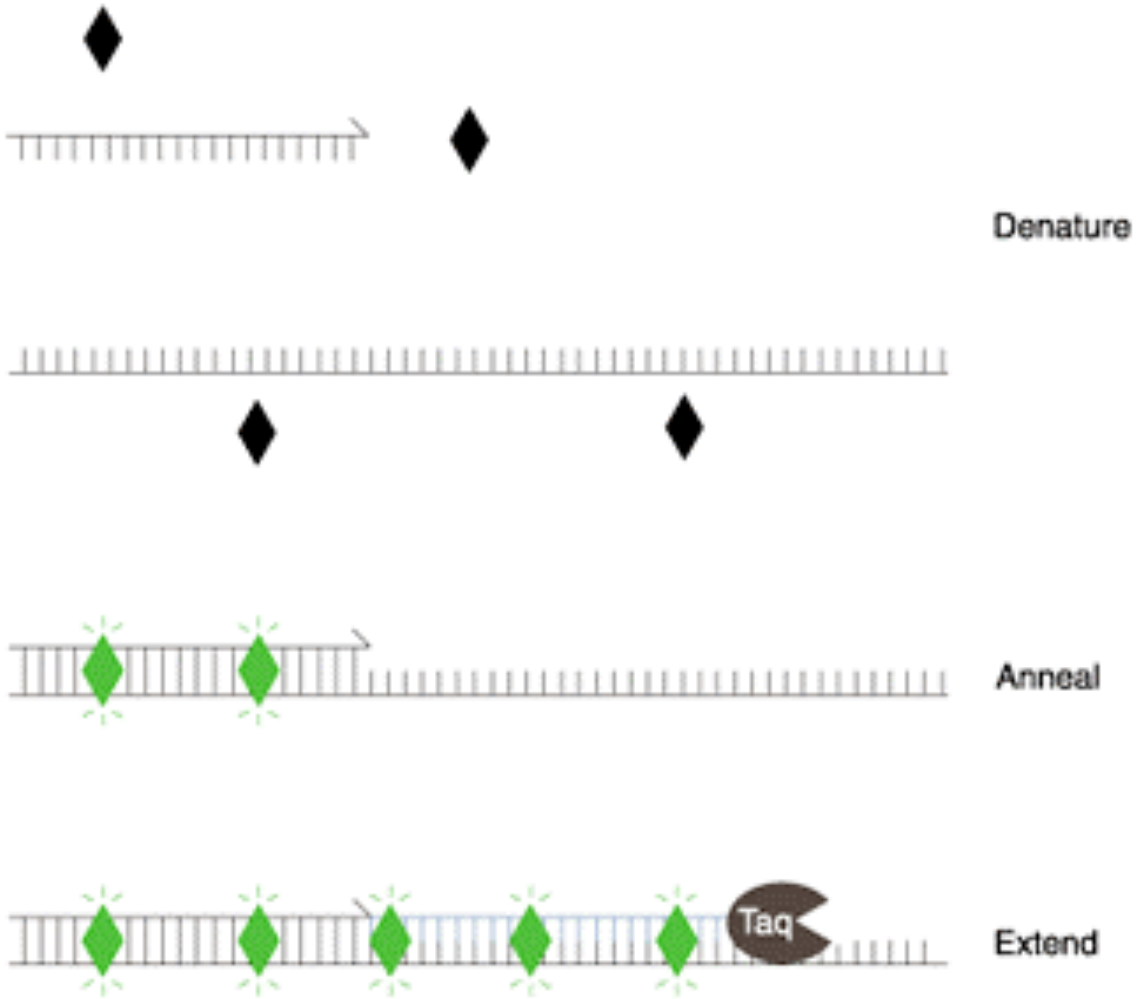


The cycle (C_t) where reaction becomes exponential is related to target copy number.

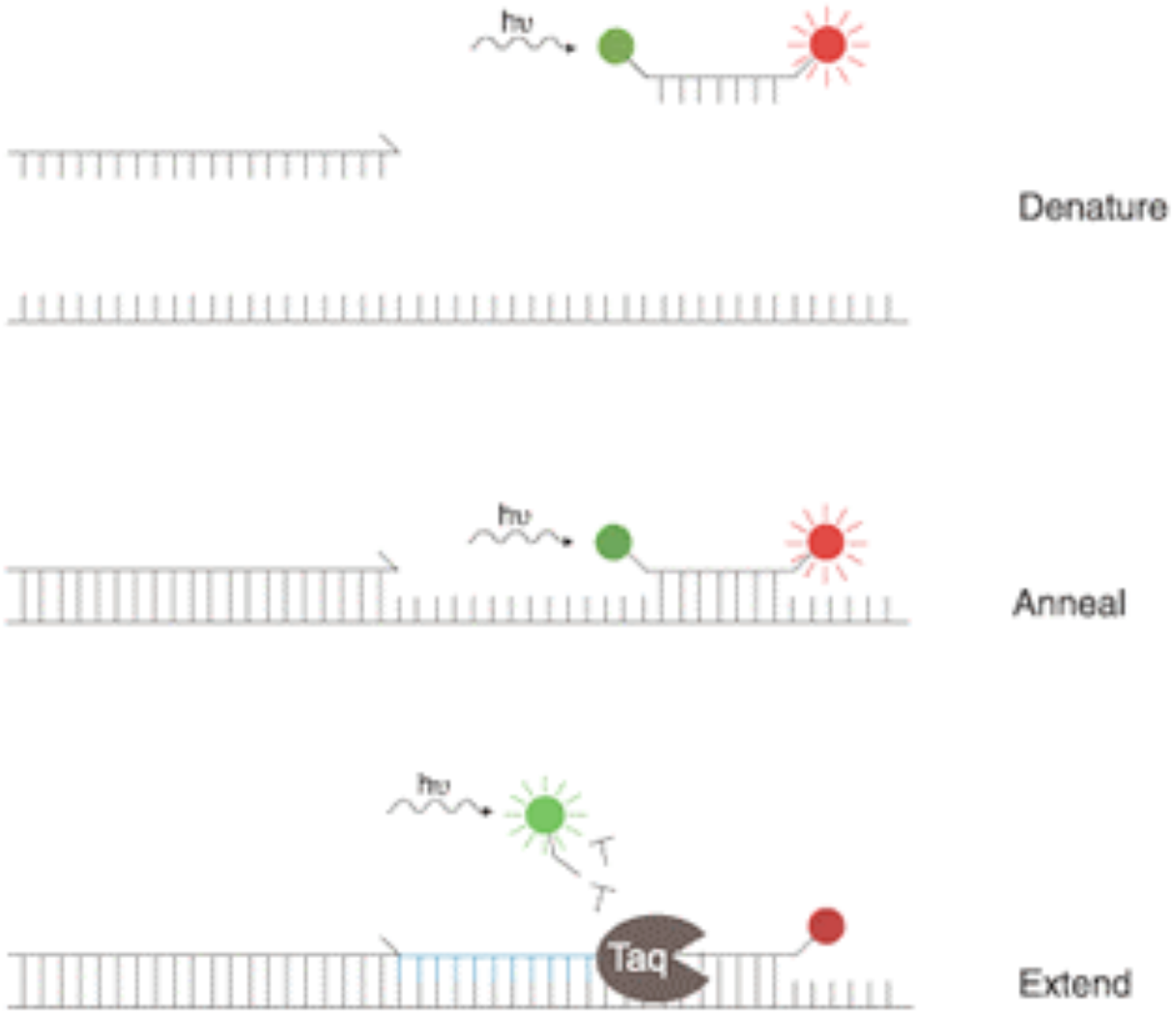
Well established in biomedical research.



SYBR® Green I Dye Chemistry



TaqMan® Probe Chemistry



In principle, larval abundance can be assessed without sorting from whole sample analysis.

Plankton Tow
(Elkhorn Slough, Monterey Bay

↓

Whole sample DNA extraction

↓

qPCR with species specific primers/probes

↓

Compare to calibration curve with known target concentrations

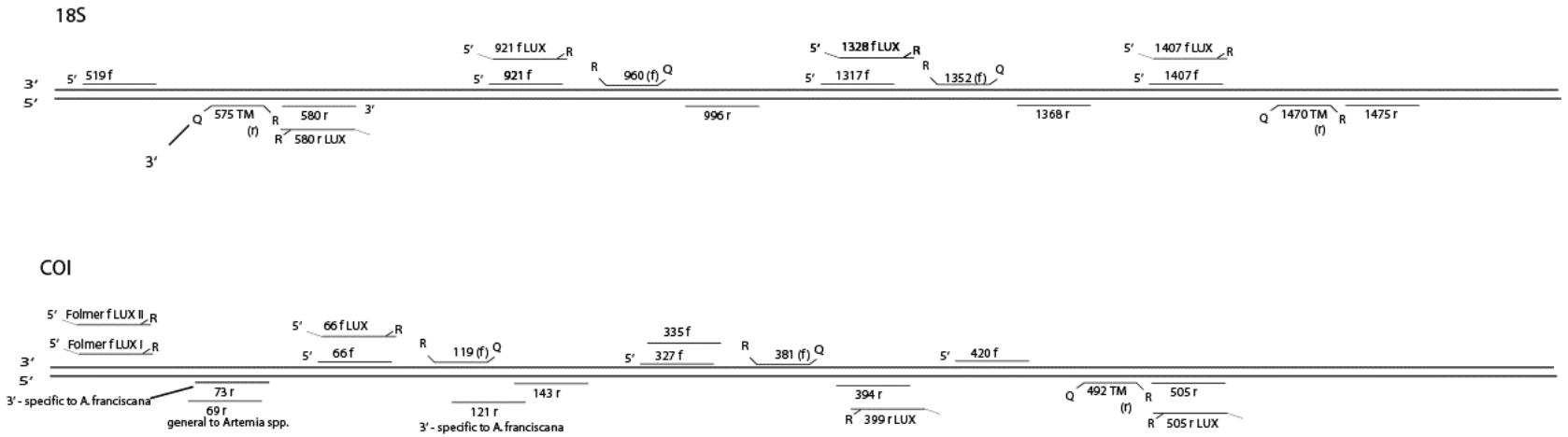
We addressed some practical issues that may complicate implementation in an environmental setting.

- How do different reporter systems perform?
- Can a single species be uniquely amplified in a background of whole plankton sample?
- How does qPCR perform in complex background of environmental DNA vs. in water alone (how does excess DNA itself affect rxn)?
- How does qPCR perform in different environmental DNA backgrounds (or different environmental contaminants)?
- What is range over which qPCR is useful (min./max. number of larvae)
- How do larvae compare to adults (in qPCR signal)?

Our approach: use *Artemia franciscanus* nauplii as test case.

Species specific primers

Primer selection scheme. Primer pairs selected for amplification *A. francsicana* COI and 18S were tested on a sample of DNA extracted from plankton.



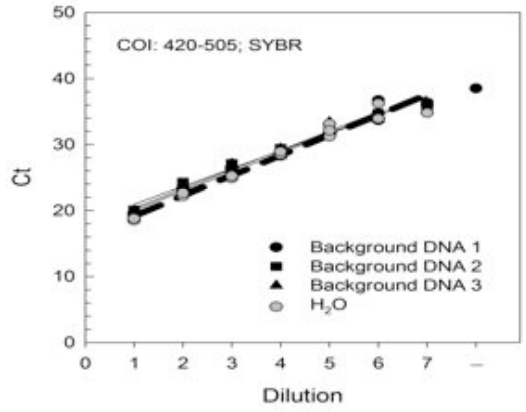
The range of primer combinations trialed in initial PCR-agarose experiments is as follows:

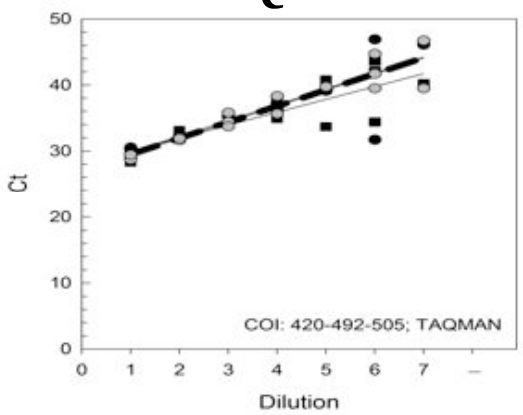
1. AF18S-519f – AF18S-580r REJECT
2. AF18S-921f-AF18S-996r REJECT
3. AF18S-1317f-AF18S-1368r OK (may have primer dimer)
4. AF18S-1407f-AF18S1475r REJECT (tentative)
5. **Af18S-1298f-Af18S-1387r** **OK** **- selected for QPCR/probe design**
6. AFCOI-66f- AFCOI-121riii OK
7. AFCOI-66f - AFCOI-143r OK
8. AFCOI-327f – AFCOI-394r REJECT
9. AFCOI-335f – AFCOI-394r OK
10. **AFCOI-420f – AFCOI-505r** **OK** **- "**

Comparison of reporter systems, background DNA, target genes, and examination of effective range of detectable larval DNA

SYBR TAQMAN

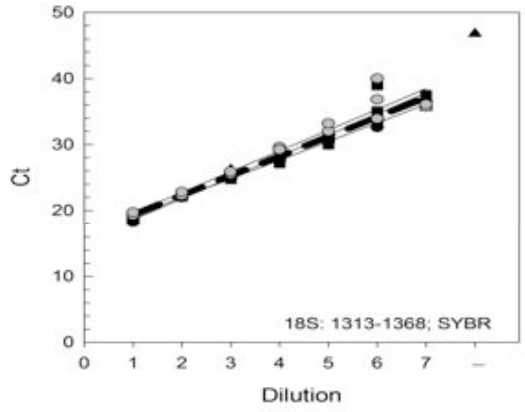
COI

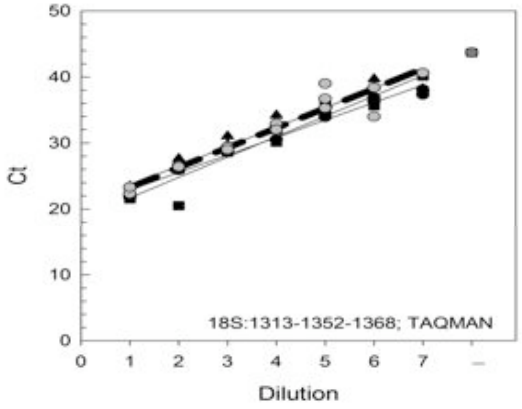




Background DNA (5 pg) had no effect on PCR vs water.

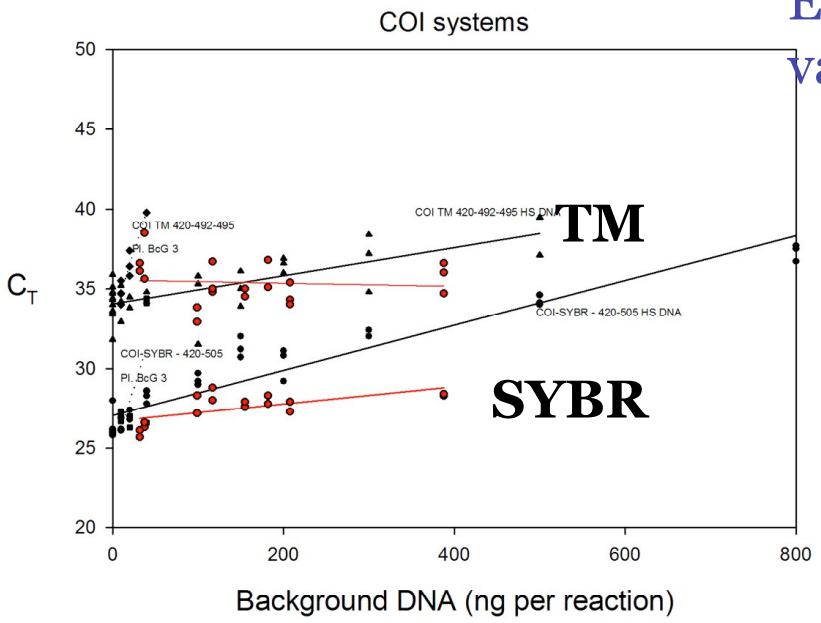
18S



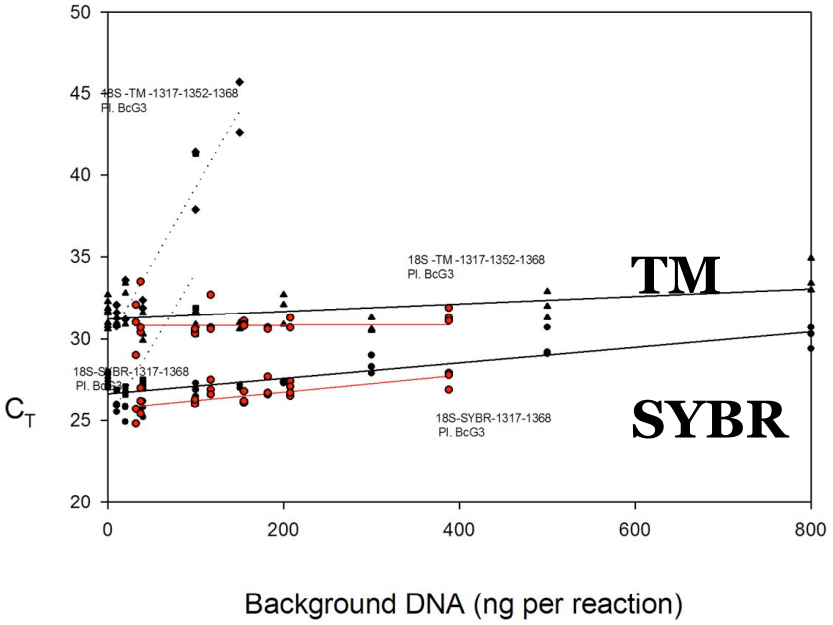


qPCR is extremely sensitive: these graphs show linearity across 7 orders of magnitude (10 ng to 10^{-6} ng) using two targets (CO1 and 18S and two reporter systems (SYBR and Taqman) in a background of 5 ng pDNA (three plankton tows). NOTE: 1 nauplius=~10 ng DNA; 10^{-6} ng=~ 10^{-5} nauplii).

Experiment: constant *Artemia DNA* (10 ng), variable backgrounds



10 ng Af DNA



High background of pDNA concentration mildly inhibits PCR (but not specificity)

Plankton DNA ● or pure herring sperm DNA ▲ was added to reactions, leading to no or slight PCR suppression in most cases.

This helps us identify a reliable range for an actual plankton sample.

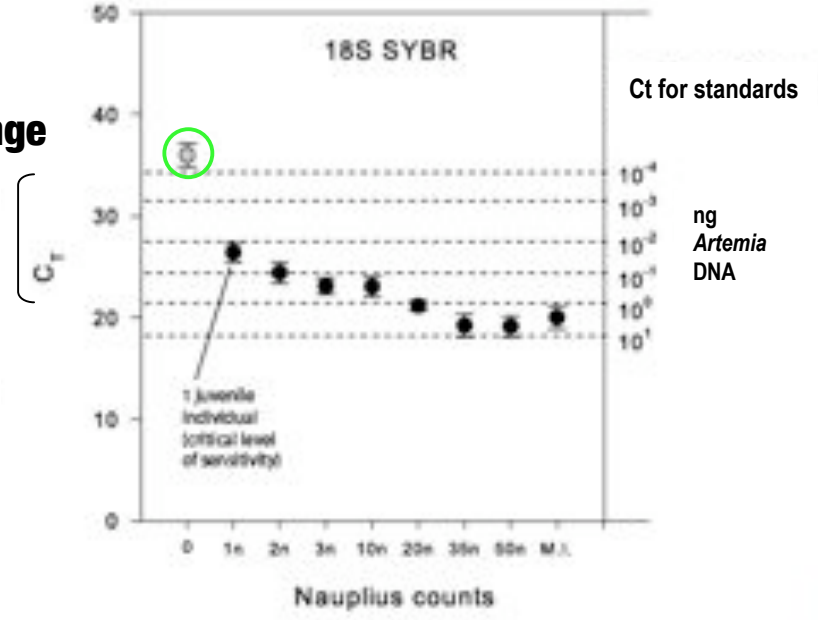
One algae rich sample of background DNA ● was a strong PCR inhibiter- likely due to contaminants rather than DNA.

SYBR was more sensitive than Taqman.

Almost the real world.....
Detecting live nauplii added to wet plankton

A single nauplius (1 ug) is detected when added to a 20 mg plankton sample
(extractions yield 123-580 ng/uL; 10 ng of extracted DNA was used in each rxn)
Signal from one adult=~35 nauplii. qPCR was linear from 1-20 nauplii/rxn
qPCR is accurate; Distinguishing order of magnitude differences in abundance looks easy

Reliable qPCR range



- How do different reporter systems perform?
  SYBR is more sensitive (and cheaper).
- Can a single species be uniquely amplified in a background of whole plankton sample?
  Yes (for *Artemia*); controls and ground-truthing necessary.
- How does qPCR perform in complex background of environmental DNA vs. in water alone.
  Complexity had no effect on specificity or efficiency. High concentration can be inhibitory.
- How does qPCR perform in different environmental DNA backgrounds (or different environmental contaminants)?
  Some plankton samples can be strongly inhibitory.
- What is range over which qPCR is useful (min./max. number of larvae).
  1-20 in 20 mg of wet plankton. Dilution of extracted DNA can take number down to 10^{-5} larvae.
- How do larvae compare to adults (in qPCR signal)?
  One adult=~50 nauplii. Result are “nauplius equivalents”

Major results

Single larva PCR will be effective for identification when used with taxon-specific primers and when a rich DNA barcode database comes online. Downside is time and labor costs.

Recommendation: use for identifying key species (eg, blooms); for diversity assessment, explore whole community analysis (e.g., emulsion PCR).

qPCR is specific, robust and sensitive. Use of *Artemia* to routinely spike marine samples to assess extraction and PCR efficiency and determine optimal conditions for each sample.

Recommendation: qPCR is ready to apply to quantitative plankton studies.