Outline

Potential Target Journals

a. *The Journal of Experimental Biology*
b. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology or Aquatic Toxicology or Chemosphere*

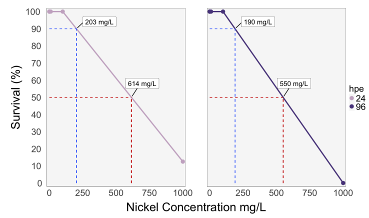
Study Narrative

- Case study in extreme adaptation? Nickel as a tool to explore stress adaptation
    - Molecularly exploring how Botryllus buffers extreme metal exposure
    - Foundational dataset exploring post-transcriptional regulation in tunicates under stress
- Why can Botryllus handle stress so well
- Nickel as a tool to **probe their molecular resilience (environmentally irrelevant dosage)**
- *Botryllus* may have unique **stress adaptation mechanisms** that other species don't and this can potentially be seen in the non-linear dose response.
    - Cite the non-linear dose response in uv-b irradiation study
- Integrating **transcriptomics and proteomics** helps uncover **post-transcriptional regulation**, which could explain how they buffer against stress without linear dose-dependence.
- DNA damage, stress response, and cell proliferation and **new insights** into how *Botryllus* reacts to environmental stressors at a molecular level,

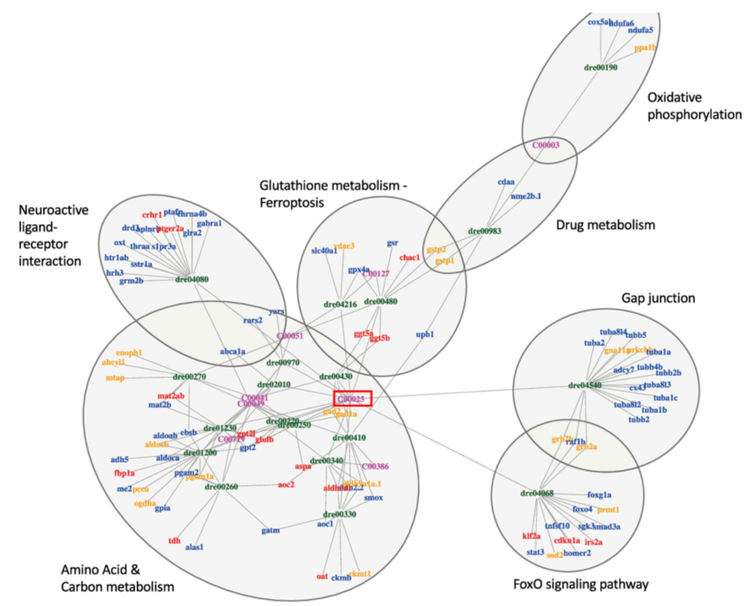
Outline

- **Introduction**: Discuss why *Botryllus* is an interesting model species for ecotoxicology, emphasizing its unique biology and relevance to nickel exposure. Briefly touch on the lack of studies exploring the molecular mechanisms of stress in *Botryllus*.
    - **Objective/Question**: This study investigates the molecular response of *Botryllus schlosseri* colonies to nickel exposure using transcriptomic and proteomic analyses to identify key pathways involved in stress response and cellular adaptation.
- **Methods**: Detail the experimental design, including the exposure concentrations, the collection and preparation of tissue, and the methodologies for RNA sequencing and proteomics analysis. Specify any data normalization or analysis methods (e.g., DESeq2 for RNAseq, statistical analysis for proteomics).
- **Results**:
    - Differentially expressed genes and proteins Include **key pathways**

        - TGF-beta signaling,
    - Figures
        - Figure 1A-B: 24 and 96hr LC50 curves



        - Figure 3: Photomicrograph of changes to Botryllus following acute NiCl exposure (representative images).
        - Figure 5: PCA for transcriptomic data
        - Figure 6: PCA for proteomic data
        - Figure 7A-B: KEGG pathway enrichment analysis of DEGs and DEPs for transcriptome and proteome
        - https://www.researchgate.net/figure/Proteo-transcriptomics-correlation-a-Pearson-correlation-analysis-between-plasma_fig2_369925156
        - Pathway networks



        - Figure 7: Heatmap with Correlation analyses between the two datasets
        - Figure: Heat map of protein coding transcripts differentially regulated between treated and untreated
        - Figure 8: GO Term Bar Plot: Relative abundance of gene ontology terms compared between treated and untreated
- **Discussion**: Address the potential **biological significance** of the upregulated/downregulated genes and proteins in response to nickel exposure.

    - **What mechanisms allow *Botryllus* to tolerate extreme stress?** (DNA repair, stress response pathways, protein turnover, cell cycle arrest, etc.)
    - **Are these mechanisms conserved in other species?** If not, does this suggest *Botryllus* has a unique way of handling toxicants?
    - **Could these mechanisms be harnessed for other applications?** (e.g., cell line development, bioremediation models, or understanding extreme stress tolerance in other organisms.)
    - Comparison of data with existing findings from other species or similar environmental stressors will make the research more impactful.
- **Conclusion**: What are the implications for further research on DNA repair pathways, cell proliferation, and environmental stress adaptation?
- **Limitations**:
    - Current paper lays the groundwork for these follow-up experiments in future studies through validation such as qPCR or Western blotting
        - Or if time permits follow this up with qPCR? At multiple timepoints? Recovery?

NiCl2xBSc Manucsript 1

Introduction

Botryllus schlosseri is a cosmopolitan marine colonial ascidian species with a near global distribution across the shallow and temperate coastal zones of all continents except Antarctica (Zwahlen et al., 2022). The first report of *B. schlosseri* along the Pacific coast of the USA dates back to 1940s within California state coast boundaries, with the population having since spread north into Washington state coasts since the late 1980s (Karahan et al., 2016). Their success as an invasive species can be attributed to a relatively high degree of phenotypic plasticity, capable of enduring the vast environmental perturbations occurring with transoceanic as a fouling organism attached to moving substrates like vessel hulls and commercially relevant aquaculture organisms (Chadwick-Furman and Weissman, 1995; Karahan et al., 2016). Colonies of *B. schlosseri* are primarily found in boat harbors and marinas, settled on both anthropogenic and natural substrata like buoy floats, docks, ships' hulls, rope, mussels, tubeworms, and seaweed.

Materials and Methods

Colony Field Collection and Husbandry

Adult *Botryllus schlosseri* colonies were collected in a series of field collections from the City of Des Moines Marina, Des Moines, WA, USA (47°23'51.18"N 122°19'46.01"W) from September to November 2024. *B. schlosseri* colonies in the City of De Moines Marina found were carefully removed from the floating docks and placed in 400 mL plastic containers containing marina seawater. Containers were placed in a cooler over the course of the collection periods. Colonies were transported to the University of Washington, Tacoma, WA, USA and immediately processed for intake into the UW Tacoma's recirculating artificial seawater system (RAS) (18 °C; pH 8.0; 30 ppt; 12:12 dark:light photoperiod). Colonies were peeled off blue mussels using a clean razor blade, being careful to not puncture the tunic or tear the extending ampullae. Colonies were then transferred to a 15 cm glass petri dish filled with marina seawater and cleaned using a soft brush under a stereomicroscope. Only visually healthy colonies were kept for lab acclimation – visually determined using the following criteria: a flat and firm tunic, normal zooid cluster formations, and at least one area or point of ampullae extension. Colonies were then tied to 76.2 mm x 50.8 mm x 1.2 mm glass slides with a cotton thread and placed back in the 400 mL vessel with marina water while all other colonies were processed. Tied colonies were then transferred into the RAS with slides maintained vertically in slots of glass staining racks. *B. schlosseri* colonies were held at a stocking density of 12 genotypes per 5-liter plastic container of the RAS. *B. schlosseri* were fed every other day a mixture of Liquid RotiRich (Florida Aqua Farms) and Reef Phytoplankton (Seachem). Colonies were cleaned with a soft brush and re-tied to their glass slides every other day or as needed to encourage gliding and adherence of peripheral systems glass slides.

For the range-finding study, *B. schlosseri* were acclimated to the RAS for 11 days prior to experimentation. At the 9 day mark, colonies with at least 2 systems adhered to the glass slide and in good observable health: extended ampullae; clear and firm tunic; normal pigmentation – were subdivided two days prior to nickel exposure using a clean razor blade into single system colonial fragments (ramets). Ramets for all range-finding experiments had an average of 11 zooids (± 4).

Colonies utilized in the RNA-seq study were acclimated, maintained, and blastogenic stage monitored in the RAS for 10 days (± 3). Colonies that had at least 2 systems adhered to the glass slide and in good observable health were divided into two ramets at blastogenic stage B. Upon maturing into blastogenic stage C1, colonies were starved prior to beginning exposures at stage C2.

Chemicals

All glassware used in experiments were triplicate washed with laboratory detergent, triplicate rinsed with tap water, and triplicate rinsed with deionized water prior to start of exposures. Glassware used in exposures included exposure vessels (473 mL wide-mouth glass mason jars), 1 L beakers for nickel solution preparations, and 15 cm glass petri dishes.

A nickel stock solution of 1000 g L^{-1} was made by suspending nickel (II) chloride hexahydrate salt (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. 339350) in Milli-Q water. Artificial seawater (ASW) (27 ppt, 8.0 pH) was prepared by dissolving Red Sea Salt (RedSea) in deionized water and aerated at 18 °C a day before exposure starts. 200 mL of ASW was added into each exposure vessel. Each vessel was spiked with the appropriate volume of nickel stock solution to achieve desired concentration. Aliquots of each solution were reserved for analytical water chemistry at the start of each experiment.

Rangefinder Study

All following experimental procedures were carried out in a controlled environmental chamber (Percival) set to 18 °C (12:12 light:dark photoperiod).

RNA-seq Study

Analytical Chemistry

- Concentrations of nickel in water were assessed with ICPMS.
    - 10 mL of water required
- Concentrations of nickel in *Botryllus* body were assessed with ICPMS
    - Mass of dried animal required to run this is TBD

LC_{50} Determination

LC50 nickel exposures began 12 days after specimen intake.

- Exposures began 12 days after intake of animals
- Only healthy animals used
- Animals starved 24 hours prior to LC50 experiment
- Animals cleaned as described below
- Animals photographed prior to exposure
- Animals distributed to exposure beakers
- 0-1000 mg/L
    - 0
    - 0.1
    - 1
    - 10
    - 100
    - 1000

- Exposure is 96 hours
- Mortality marked by the cessation of blood flow in the system and surrounding ampullae.

Experimental Nickel Exposures

Nickel exposures were staggered. Only colonies with a minimum of 90 zooids embedded in tunic adhered to the glass slide, healthy with extending ampullae, good coloration, and clear tunic were used in the trials. At stage B2, animals were divided from the rest of the mother colony that had failed to adhere to glass slides. Animals were starved 24 hours prior to the commencement of exposures. Prior to exposure start, animals were removed from the recirculating system and brushed with a soft paintbrush to remove excess debris. A clean stainless steel razor blade was used to clean off accumulated biofilm from the surrounding glass slide. Exposures began when colonies reached blastogenic stage C2 to allow them to reach the final blastogenic stage D over the course of the exposure. After 24 hours of exposure, animals were removed from the exposure system and rinsed thoroughly with 50 mL of artificial seawater (27 PPT). Colonies were then microdissected for their stage D primary buds with a pair of 28 G insulin needles and microdissection forceps. 50 primary buds were allocated to proteomics and 50 were allocated to RNA extractions.

- Animals starved 24 hours prior to commencement of exposures.
- Animals were removed from the recirculating system and brushed with a soft paintbrush to remove excess debris. A razor blade used to clean off the glass slide.
- Animals were photographed with exon stereomicroscope.
- Beakers were filled with 250 mL of exposure water.
- Glass slides with adhered animals were gently placed into the exposure beakers.

- Exposure beakers were parafilmed and perforated and placed back into environmental chamber for remainder of exposure
- After 24 hours, animals were removed from the exposure beakers and thoroughly rinsed with ASW to terminate exposure.
- Control and 100 mg/L treatment.

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Statistical Analyses

Results

Discussion

References

Chadwick-Furman, N. E. and Weissman, I. L. (1995). Life Histories and Senescence of *Botryllus schlosseri* (Chordata, Ascidiacea) in Monterey Bay. *Biol. Bull.* **189**, 36–41.

Karahan, A., Douek, J., Paz, G. and Rinkevich, B. (2016). Population genetics features for persistent, but transient, Botryllus schlosseri (Urochordata) congregations in a central Californian marina. *Mol. Phylogenet. Evol.* **101**, 19–31.

Zwahlen, J., Reem, E., Douek, J. and Rinkevich, B. (2022). Long-term population genetic dynamics of the invasive ascidian Botryllus schlosseri, lately introduced to Puget Sound (Washington, USA) marinas. *Estuar. Coast. Shelf Sci.* **270**, 107840.