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Variable allelopathy among phytoplankton reflected in red tide metabolome

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ABSTRACT

Harmful algae are known to utilize allelopathy, the release of compounds that inhibit competitors, as a form of interference competition. Competitor responses to allelopathy are species-specific and allelopathic potency of producing algae is variable. In the current study, the biological variability in allelopathic potency was mapped to the underlying chemical variation in the exuded metabolomes of five genetic strains of the red tide dinoflagellate *Karenia brevis* using ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The impacts of *K. brevis* allelopathy on growth of a model competitor, *Asterionellopsis glacialis*, ranged from strongly inhibitory to negligible to strongly stimulatory. Unique metabolomes of *K. brevis* were visualized as chemical fingerprints, suggesting three distinct metabolic modalities – allelopathic, non-allelopathic, and stimulatory – with each modality distinguished from the others by different concentrations of several metabolites. Allelopathic *K. brevis* was characterized by enhanced concentrations of fatty acid-derived lipids and aromatic or other polyunsaturated compounds, relative to less allelopathic *K. brevis*. These findings point to a previously untapped source of information in the study of allelopathy: the chemical variability of phytoplankton, which has been underutilized in the study of bloom dynamics and plankton chemical ecology.

1. Introduction

Allelopathy, the release of compounds that inhibit competitors, is a form of interference competition that is well documented in terrestrial plants (Nilsson, 1994; Ridenour and Callaway, 2001), zooplankton (Folt and Goldman, 1981), and phytoplankton (Fistarol et al., 2004; van Donk and van de Bund, 2002). Whereas some previous authors have used a broad scope for allelopathy including positive interactions as well as those mediating interactions among organisms beyond competitors (Rice, 1984), the current work emphasizes allelopathy as a chemically mediated process of competition for limited resources among planktonic microalgae consistent with Legrand et al. (2003). Allelopathy is one of the mechanisms hypothesized to affect harmful algal bloom (HAB) dynamics (Smayda, 1997), although the role of allelopathy in the formation of HABs has been disputed (Jonsson et al., 2009). While some HAB-forming species produce potent toxins such as brevetoxins (Baden, 1989), domoic acid (Bates et al., 1989), and saxitoxins (Maranda et al., 1985), none of these toxic compounds have also been identified as allelopathic agents; one exception is the karlotoxins which have been shown to inhibit competitor growth and immobilize prey with effects depending on sterol composition of target species (Sheng et al., 2010).

Typically, in allelopathy research, scientists have sought to identify allelopathic compounds by applying bioassay-guided fractionation to an exudate of a potently allelopathic organism (Ma et al., 2009; Poulson et al., 2010; Prince et al., 2010). This has left untapped potentially useful biological and chemical variability among samples, whether cultivated or field-collected, of an allelopathic species. Recent advances in metabolic profiling have led to the emergence of chemical fingerprinting as a tool for taxonomic analysis and authentication (Choi et al., 2005; Kim et al.,

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2011; Kim et al., 2005), disease diagnosis (Ellis et al., 2007; Ellis and Goodacre, 2006; Lamers et al., 2003), and classification of multi-state systems, such as stressed vs. non-stressed organisms (Johnson et al., 2003; Poulson-Ellestad et al., 2014). Within-species metabolic variability has also been leveraged to guide identification of mating pheromones in phytoplankton (Gillard et al., 2013), virulence-regulating small molecules in worms (Bommarius et al., 2013), and enzymes responsible for the biosynthesis of nematode pheromones (von Reuss et al., 2012).

The HAB-forming dinoflagellate *Karenia brevis,* colloquially known as Florida red tide, produces a family of sodium channel agonists called brevetoxins which do not act as allelopathic compounds (Kubanek et al., 2005; Prince et al., 2010). Allelopathy by *K. brevis* is mediated by unknown compounds exuded by cells during blooms, understood to negatively impact competing phytoplankton, especially diatoms such as *Amphora* sp., *Skeletonema grethae* (formerly *S. costatum), Thalassiosira* sp., and *Asterionellopsis glacialis* (Kubanek et al., 2005; Prince et al., 2008a). Competitor sensitivity to *K. brevis* allelopathy is variable among competitor species, with some species exhibiting resistance to allelopathic compounds and others suffering reduced growth rates, longer lag before the onset of exponential growth, membrane integrity loss, compromised photosynthesis, inhibited osmoregulation, and increased oxidative stress (Poulson-Ellestad et al., 2014; Prince et al., 2008a, 2008b). Previous attempts to identify *K. brevis* compounds responsible for allelopathy have failed; however, chromatographic fractions containing allelopathic compounds exhibited spectroscopic features consistent with aromatic compounds and unsaturated hydrocarbons (Prince et al., 2010). Due to the substantial variability in allelopathy in this system, it seemed reasonable to hypothesize that variation in allelopathic potency is also reflected in the exuded metabolome of *K. brevis.* In the current study, nuclear magnetic resonance (NMR) spectroscopy-based comparative metabolomics was used to fingerprint *K. brevis*, correlating allelopathic potency with chemical variability of exuded metabolomes.

2. Materials and methods

2.1. Phytoplankton culturing

Five strains of *Karenia brevis* were obtained from the National Center for Marine Algae and Microbiota (CCMP 2228, CCMP 2229, CCMP 2281) or Lisa Campbell at Texas A&M University (TxB3 and TxB4) and cultured in L1 growth media formulated with Instant Ocean at 35 ppt. The model diatom, *Asterionellopsis glacialis* (CCMP 137), a naturally co-occurring competitor known to be moderately vulnerable to *K. brevis* allelopathy (Kubanek et al., 2005; Prince et al., 2008a), was cultured using the same conditions. Cultures were subjected to a 12:12 light:dark cycle, with irradiance 75–120 μmol m^{-2} s^{-1} at a temperature of 22 °C. Growth of *A. glacialis* was approximated using in vivo chlorophyll a fluorescence (Turner Biosystems Trilogy Fluorometer).

2.2. Allelopathy assay and generation of extracellular extracts

To determine allelopathic potencies of batches of each of the five *K. brevis* strains, 12 biological replicates of each strain were cultured in full strength L1 media in paired flasks from the same inoculum: one large 1.8 L culture for the collection of exuded allelochemicals and a second, 250 mL flask that contained a co-culture with *A. glacialis* to assess allelopathic effects. Allelopathy assay flasks consisted of *K. brevis* grown in a 50 mL falcon tube cage (ends removed and capped with 1 μm nylon mesh) placed in a culture of *A. glacialis.* The diatom *A. glacialis* was grown in dilute L1 medium (90% of typical sodium phosphate levels, 65% of typical nitrate levels, plus typical concentrations of vitamins, and trace metals) in the presence of the same cage acted as a control in the allelopathy assay, to mimic resource consumption by *K. brevis* which was found to deplete macronutrients as described above (Poulson-Ellestad et al., 2014). To make the set-up manageable, the 12 replicate cultures of each strain were split into three batch experiments (blocks) that were performed sequentially, with each block containing four replicates of each strain. Thus, the experiment was designed to assess both strain and batch variability in allelopathic compound production.

Growth of the *K. brevis* was monitored every other day (starting on the first day) using samples preserved with Lugol's solution and measured using a FlowCAM autoimager (Fluid Imaging Inc.; 100 μm flow cell, 0.4 mL min^{-1}, autoimage rate of 16 frames per second). Cell concentrations of *A. glacialis* in the allelopathy assay flasks were monitored daily via in vivo fluorescence. Growth was calculated via:

$$\%Growth = \frac{final\ fluorescence - initial\ fluorescence}{initial\ fluorescence} \times 100.$$

Strain and block effects of *K. brevis* allelopathy on percent growth of *A. glacialis* were analyzed using two-way analysis of variance (ANOVA) with PRISM version 10.0.

Cultures were grown for 10 days by which time the *K. brevis* had reached mid-exponential growth phase, at which point hydrophobic resins (a 1:1 mixture of Diaion HP20 and Amberlite XAD-7) were added to *K. brevis* monocultures (1.8 L flasks) to extract compounds exuded by *K. brevis*, as described in Prince et al. (2008a). Resins were incubated for 24 h, gently rinsed with filtered artificial seawater to remove *K. brevis* cells, and rinsed with deionized water to remove salts. Organic compounds were eluted from resins with LC/MS grade methanol and solvent was removed by rotary evaporation. The resulting extracts were triturated three times with ice-cold methanol to remove excess salts and dried again. Extracellular extracts were stored dry at −80 °C prior to spectroscopic analysis.

2.3. Allelopathic index calculation

To assign strain and batch potency of *K. brevis* allelopathy, growth of the competitor *A. glacialis* when co-cultured with *K. brevis* was used as an inverse proxy for *K. brevis* allelopathic potency. More potent *K. brevis* cultures reduced growth of *A. glacialis* and as such allelopathic index was calculated as:

$$Allelopathic\ index = \left(1 - \frac{avg\ \%\ growth\ of\ control\ for\ block - \%\ growth\ of\ A.glacialis\ in\ flask}{avg\ \%\ growth\ of\ control\ for\ block}\right) \times 100$$

Cultures of *K. brevis* with an allelopathic index within one standard deviation of the mean growth (mean growth = 115% relative to *A. glacialis*-only control, standard deviation = 79.4%) were deemed “non-allelopathic”; those more than one standard deviation below the mean (i.e., growth below than 35.2% relative to *A. glacialis*-only controls) were deemed “allelopathic”; and those more than one standard deviation above the mean (growth greater than 194.1% relative to *A. glacialis*-only controls) were deemed “stimulatory.” This assignment allowed a preliminary basis for comparison among strains and blocks, in order to test whether concentrations of some exuded metabolites were associated with a category of allelopathic potency.

2.4. NMR spectroscopic data acquisition and processing

To explore the chemical constituents of *K. brevis* exuded metabolomes, extracellular extracts from *K. brevis* monocultures