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The prevention of saltwater algal pond contamination using the electron transport chain disruptor, rotenone

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ABSTRACT

Rotenone is a classic inhibitor of Complex I of the electron transport chain which is common to all life. Algae are known to be rotenone-insensitive because in addition to Complex I they have alternative pathways for the oxidation of $NADH_2$. It is this differential sensitivity to rotenone that can be exploited to reduce many types of predators while not affecting biodiesel-producing algae. A universal tool like rotenone is needed to reduce predation in open ponds as predation is well known to limit cultivation to a few weeks in the warmer months. In this study, we tested the effect of rotenone on the mortality and reproduction of two marine algae strains, *Nannochloropsis oculata* and *Tetraselmis suecica*, and two marine rotifer strains, *Brachionus manjavacas* and *Brachionus rotundiformis*. The LC_{50}s for the two rotifers ranged from 0.18 to 0.35 μM while *N. oculata* was unaffected to at least 7.6 μM. Rotifer reproduction was reduced at rotenone concentrations lower than those that caused mortality. Rotenone applications to produce 1 million gallons of biodiesel in one year are estimated to cost $1684. This provides algae farmers an important and inexpensive tool to manage grazing predators in algae mass cultures and avoid pond crashes.

1. Introduction

Rotenone is a pesticide and a close structural homologue to the electron carrier ubiquinone found in Complex I of the respiratory mitochondrial electron transport chain (ETC) common to all life (Fig. 1). The reduction of ubiquinone is one of the first steps in the oxidation of $NADH_2$ used to pump protons and produce ATP. Rotenone binds to the ubiquinone binding site of Complex I and is an ETC inhibitor. In addition to Complex I, algae have alternative pathways for the oxidation of $NADH_2$ and the pumping of protons and are thus less sensitive to rotenone than organisms that may predate on algae [1]. It is this differential toxicity to rotenone that can be exploited to protect algae-biodiesel ponds from predation. As part of the Algae Testbed Public Private Partnership (ATP^3), the open pond cultivation of algae has been limited to a few weeks in the warmer months due to contamination which could be avoided through the use of rotenone.

Rotenone is environmentally and toxicologically safer than many of the currently used pesticides [2]. The indigenous people of French Guiana used rotenone-containing plants to kill fish which were subsequently consumed. Bleach, copper, and quinine have all been used to protect algae ponds from predation and rotenone appears to be more protective at lower concentrations [3–6]. The persistence of rotenone in the environment is well known and is a function of pH, temperature, and light [7]. Rotenone can persist for as long two weeks or more which is sufficient time to control predation in algae biodiesel ponds [2]. Although rotenone is a well-known pesticide, little research is available regarding the toxic effect of rotenone on aquatic algal predators. Salk [8] concluded that rotenone inhibited *Stichococcus chodati*, Chlorococcum, and *Bracteacoccus* algal isolates while *Chlorella* and *Klebsormidium flaccidium* algal isolates were unaffected. However, Salk [8] tested rotenone concentrations as high as 50 mg/L which may be too cost prohibitive to use in algal biodiesel ponds. Thus, we tested rotenone concentrations as low as 1.1 μg/L.

The aim of this study was to find the concentration of rotenone that can significantly inhibit algal predators without having an ill effect on algae growth. Although there are many organisms that prey on algae, they all respire using the ETC and may not have the rotenone-insensitive alternate $NADH_2$ oxidation pathways that algae do. Thus, this

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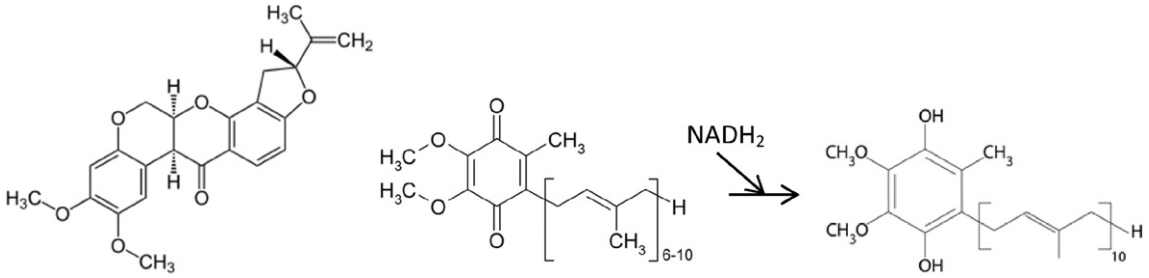

Fig. 1. Structures of rotenone (left) and oxidized and reduced forms of ubiquinone (right). Note the similar dual methyl ester ends of both molecules.

study used rotifers as a model organism to study rotenone inhibition. Rotifers are suitable model algal predators because of their microscopic size and ease of use, genetic uniformity, and high sensitivity to toxic chemicals [9]. For these reasons, the marine rotifers, *Brachionus manjavacas and Brachionus rotundiformis*, were used as model grazers to understand how to efficiently control algal predation. Thus, the research was conducted in three stages. First, acute rotenone toxicity tests were conducted to find the 24 h-LC_{50}s of the marine rotifers, *B. rotundiformis* and *B. manjavacas* and two green algae, *Nannochloropsis oculata* and *Tetraselmis suecica*. Second, reproductive toxicity tests were conducted to determine the reduction in the number of offspring produced by these rotifers. Third, *N. oculata* and *B. manjavacas* were co-cultured to determine what rotenone concentration is needed to significantly reduce predation without significantly reducing algae growth.

2. Materials and methods

The marine algae *N. oculata* and *T. suecica* were obtained from the University of Texas at Austin algae collection (UTEX #2164 & #2286, respectively, Austin, TX). The marine rotifers *B. rotundiformis* and *B. manjavacas* were originally collected in Hawaii and the Azov Sea region [10]. The alga media was f/2 media which contained the following stocks (per L of distilled water): 1) 85 g $NaNO_3$ and 7.5 g NaH_2PO_4; 2) 9.8 g $CuSO_4 \cdot 5H_2O$; 3) 6.3 g $Na_2MoO_4 \cdot 2H_2O$; 4) 22.0 g $ZnSO_4 \cdot 7H_2O$; 5) 10.0 g $CoCl_2 \cdot 6H_2O$; 6) 180 g $MnCl_2 \cdot 4H_2O$. A trace metal stock solution (per L of distilled water) was made by first dissolving 4.36 g $Na_2EDTA \cdot 2H_2O$, then adding 3.15 g $FeCl_3 \cdot 6H_2O$ and 1 mL of stocks 2–6. The culture media (per liter) was made using 35 g of Instant Ocean sea salt, 9 mL of stock 1, and 1 mL of the trace metal stock. Reagent grade chemicals were used. The optical density (OD) of algal suspensions was obtained at 750 nm using a Thermo Spectronic Genesys 20 spectrophotometer (USA).

Rotifer eggs were hatched in sterile Petri dishes containing spring water containing 15 g/L Instant Ocean sea salt after 16 h in a Barnstead Lab-line 305 Imperial III incubator (IL, USA), maintained at 25 °C with continuous light from a 6-Watt (40 W) warm white LED light bulb (2700 K, 450 Lumens) with a vertical light path of ~8 in. Rotifers were counted using a stereomicroscope (SMZ-2T, Nikon Co., Tokyo, Japan).

2.1. Acute and reproductive rotenone toxicity tests of rotifers

Acute toxicity test methods with rotifers have been standardized by Snell et al. [11]. Briefly, a 500 mg/L rotenone stock solution was prepared by dissolving 10 mg rotenone into 20 mL of dimethyl sulfoxide. With each rotifer, three experiments were conducted with four replicates at 4–10 different rotenone concentrations ranging from 0.004 to 25 μM by serial dilution in 24-well plates at 1 mL per well. Wells were covered to prevent evaporation. Ten to twelve rotifer neonates were transferred into each well and were incubated for 24 h. The well plates were examined under the stereomicroscope for rotifer mortality after 24 h to calculate LC_{50}'s. The number of live and dead rotifers was recorded, with rotifers not moving for 10 s regarded as a dead. Reproductive toxicity tests were conducted in a similar manner except three concentrations were tested ranging from 0.005 to 0.3 μM, the tests were initiated with 24 rotifers, and the number of offspring was counted after 48 h.

2.2. Rotenone toxicity tests using N. oculata and T. suecica

Based on the LC_{50} obtained from the acute rotifer toxicity tests, marine algae suspensions were spiked with rotenone at 0 to 1.0 μM. Tests were conducted in triplicate. ODs were measured over 8 days to determine the growth rate of algae cells.

2.3. The toxicity of rotenone to B. manjavacas in N. oculata suspensions

Stock cultures of *N. oculata* were grown in 1 L glass tubes (5.1 cm wide, 0.64 m tall) with a conical bottom, were given continuous illumination from the side by 12 40-Watt 1.2 m long cool white fluorescent light bulbs with a light path of ~3 in., and sparged with air at ~1 L/min. Log-phase algal suspensions (100 mL) were then used to initiate 250 mL Erlenmeyer shake flask batch experiments in which we added

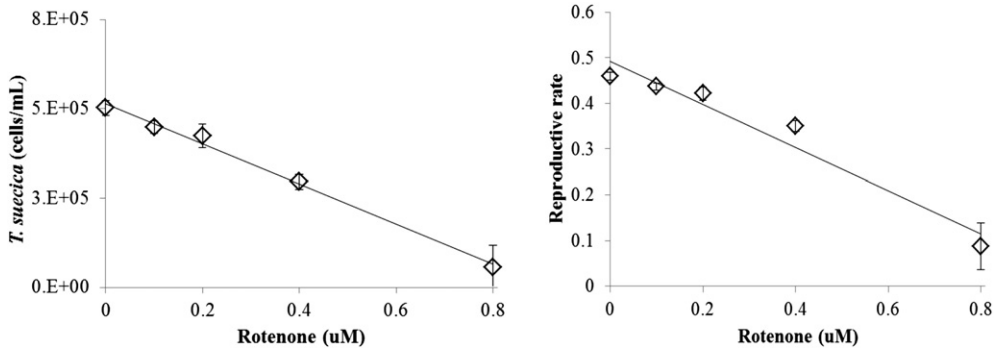

Fig. 2. Effect of rotenone on *T. suecica* growth and reproductive rate.