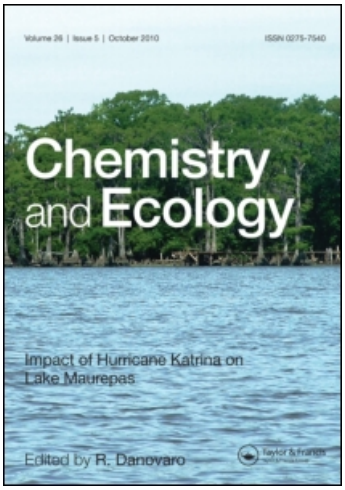

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Oxyradical scavenging capacity by the S9 fraction of *Hexagenia* spp. nymphs from the Western Basin of Lake Erie: neutralisation of three potent oxidants

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Oxidative stress is a general response of aquatic organisms to environmental contamination. Metals and organic compounds capable of redox cycling cause proliferation of reactive oxygen species (ROS) within organisms. Harm results from ROS-mediated DNA damage, lipid peroxidation, enzyme degradation and compromised intermediary metabolism. Variations in concentrations or activities of antioxidants have been proposed as biomarkers of toxicant-mediated oxidative stress in aquatic organisms. The total oxyradical scavenging capacity (TOSC) assay provides an index of biological resistance to ROS. Burrowing mayflies (*Hexagenia* spp.) are key indicator species of the health of numerous water bodies, including Lake Erie. TOSC has been used to evaluate the ROS scavenging capacity of tissues from a number of marine and freshwater invertebrates. This is the first study to evaluate ROS scavenging capacity in *Hexagenia* nymphs. *Hexagenia* nymphal tissue was homogenised in TRIS buffer containing a protease inhibitor cocktail and then differentially centrifuged to obtain a 9000 *g* supernatant (S9). The specific TOSC values ($\pm$SD, $n = 3$) per μg protein are 2.08 ± 0.43 for peroxyl radicals, 3.06 ± 0.19 for hydroxyl radicals and 0.36 ± 0.02 for peroxynitrite ($n = 3$ to 4 determinations). These values for peroxyl radical scavenging capacity were equivalent to 11.9% and 77.0% that of Trolox (a water soluble analogue of vitamin E) and reduced glutathione TOSC equivalents, respectively, on a per μg basis. These results show that *Hexagenia* nymphal S9 is capable of neutralising peroxyl radicals and hydroxyl radicals effectively, but neutralises peroxynitrite considerably more weakly. The efficacy of this parameter as a biomarker of exposure to or effect of environmental contaminants will require controlled exposure analysis.

Keywords: *Hexagenia*; antioxidant; TOSC assay; peroxyl radical; hydroxyl radical; peroxynitrite

1. Introduction

Reactive oxygen species (ROS) are formed in all aerobic organisms in connection with normal oxygen reduction metabolism via various metabolic pathways. Among these are the four-electron reduction of molecular oxygen to water by the mitochondrial electron transport chain, which is coupled to oxidative phosphorylation and ATP production, microsomal or photosynthetic electron transport chains, active phagocytosis and the activities of several enzymes that produce ROS as

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intermediates [1–3]. Primary ROS produced in these cellular processes include superoxide anion (O_{2^-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^\bullet$); and secondary ROS that require metabolism beyond direct electron reduction of oxygen are peroxyl radicals ($ROO^\bullet$), alkoxyl radicals ($RO^\bullet$) and peroxynitrite (HOONO). All are strong oxidants; however, their reactivity towards biological macromolecules varies greatly, thereby impacting their toxicity. Hydroxyl radical and superoxide anion, respectively, are regarded as the strongest and weakest of these oxidants [4]. The formation of ROS intracellularly does not always have toxic ramifications because their damaging effects on biological tissues may be counteracted by various water- and lipid-soluble low molecular weight antioxidants and specially adapted enzymes. Oxidative stress is repressed if antioxidant defences are not exceeded by prooxidant challenges [5]. Free radical biology and oxidative stress are of increasing interest to the field of ecotoxicology for their applications in biomonitoring programmes [6–8]. Because environmental pollutants can enhance intracellular formation of ROS, variations in the levels or activities of the main antioxidants have been proposed as biomarkers of contaminant-mediated oxidative stress in marine organisms [5,8–11].

A common pathway of toxicity induced by a wide range of environmental pollutants is the enhancement of intracellular generation of reactive oxygen species (ROS) [5,9,10]. Variations in the levels and activities of the main antioxidants have been shown to be useful biomarkers of contaminant-mediated oxidative stress in freshwater and marine organisms [10,12].

The susceptibility of aquatic organisms to oxidative stress has classically been based on the measurement of specific, single antioxidants, their biosynthetic pathways, intracellular localisation, modes of action and responses to different stressors [12–15]. These approaches have certainly aided understanding of environmentally-induced oxidative stress; however, the complexities associated with predicting the consequences of environmental alteration on adaptation to prooxidant challenge and antioxidant defences do not provide a holistic understanding of the antioxidant capacity of organisms in quantifiable terms with respect to oxidant scavenging potential.

The total oxyradical scavenging capacity (TOSC) assay [16] measures and quantifies the capability of biological samples to neutralise ROS, thus providing an index of specific biological resistance to various kinds of ROS [17]. In the TOSC assay different ROS are generated at a constant rate in the presence of a substrate, α-keto-γ-methiolbutyric acid (KMBA), which upon oxidation by ROS produces ethylene gas. The course of ethylene formation is measured over time and the efficiency of cellular antioxidants as scavengers of the generated ROS is determined by their ability to inhibit the reaction between ROS and KMBA. Inhibition of ethylene formation relative to controls yields a TOSC value, which is an index of the antioxidant capacity of a sample for a defined ROS.

Since the relative efficiency of antioxidants may vary considerably towards different ROS, the TOSC assay has been standardised for measuring scavenging capacity with respect to various ROS [17] including peroxyl radicals, hydroxyl radicals and peroxynitrite or oxidants generated from the decomposition of peroxynitrite [18]. The TOSC assay has been used by our laboratory and that of several others to evaluate the scavenging capacity of tissues from different marine invertebrates towards various ROS [8,10,12]. Knowledge of the susceptibility of organisms to oxidative stress is of particular importance in environments in which characterisation of the responses of antioxidant defences in key indicator species may be of value as early detection biomarkers of the effects of anthropogenic activities.

Mayflies constitute an important group of sediment-dwelling organisms in water bodies throughout the world, including rivers, streams, ponds and shallow lakes [19]. Mayfly nymphs of some species live their entire existence in underlying water sediments until their emergence as flying sub-adults (sub-imagoes) that quickly molt into adults (imagoes). Mayfly nymphs are a vital link in the food web of freshwater ecosystems, making energy stored in bacteria, algae and detritus available to higher consumers (other invertebrates, fish, birds, etc.). These insects

are routinely used for monitoring water quality because their presence and diversity can be valuable indicators of the health of their aquatic environment. In North America, mayfly species are susceptible to existing and impending environmental degradation [20].

Considering the importance of biological responses, which may be used for rapid detection of both anthropogenic and natural disturbance to *Hexagenia* nymphs in Lake Erie, and the sensitivity of antioxidant responses for predicting short term effects induced by stressful conditions, the aim of this work was to characterise ROS absorbance capacity, an important antioxidant defence parameter, to gain perspective on the ability of *Hexagenia* nymphs to withstand oxidative stress. In this respect, the TOSC towards peroxyl radicals, hydroxyl radicals and peroxynitrite was investigated in *Hexagenia* spp. nymphs from the western basin of Lake Erie to establish baseline values for future work on exposure and for comparative biochemical purposes.

2. Materials and methods

2.1. *Collection and maintenance of* Hexagenia *nymphs*

Hexagenia nymphs (∼20–30 mm in length) were collected from the western basin of Lake Erie with an Ekman grab sampler. Approximately 30 nymphs were obtained, transferred to the laboratory in buckets of sediment and water, and kept in glass aquaria with an 8 cm layer of Lake Erie sediment and a 6–8 cm layer of Lake Erie water. The water was constantly aerated and the nymphs were acclimated for 3 weeks before homogenising for biochemical analysis.

2.2. *Preparation of* Hexagenia *S9 fraction*

Nymphs were collected from the sediments by sieving and placed in liquid nitrogen for 1-hour transport by automobile to the Department of Biological Sciences at Bowling Green State University, Ohio. There, the nymphs were weighed, diced and homogenised with a Potter-Elvehjem homogeniser in ice-cold TRIS-EDTA buffer (pH 7.4) containing 5 μg/mg wet weight nymphal tissue of a protease inhibitor cocktail. The homogenate was centrifuged at 500 *g* in a Sorvall RC 5B refrigerated centrifuge in an SS-34 rotor to remove cytoskeletal material and tissue debris. The supernatant was collected and spun again at 9000 *g* in an SS-34 rotor. This supernatant was aliquot into Eppendorf tubes and stored at −30 °C until used in the TOSC assay.

2.3. *TOSC assay*

For the TOSC assay [16], peroxyl radicals, hydroxyl radicals and peroxynitrite were generated independently: thermal homolysis of 2-2′-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) was used for peroxyl radicals, the iron-ascorbate Fenton reaction for hydroxyl radicals, while peroxynitrite was generated from 3-morpholinosydnonimine HCl (SIN-1), a molecule that releases concomitantly nitric oxide and superoxide anion, which rapidly combine to form HOONO [18]. The reactions were conducted in 15 mL vials capped with Mininert® valves (Supelco, Bellefonte, PA) at 25 ± 1 °C with constant shaking in a Precision reciprocal shaking water bath (Thermo Fisher, Waltham, MA). Assay conditions were: (a) 0.2 mM KMBA, 20 mM ABAP in 100 mM potassium phosphate buffer, pH 7.4, for peroxyl radicals; (b) 1.8 μM $FeCl_3$, 3.6 μM EDTA, 0.2 mM KMBA, 180 μM ascorbic acid in 100 mM potassium phosphate buffer, pH 7.4 for hydroxyl radicals; and (c) 0.2 mM KMBA and 80 μM SIN-1 in 100 mM potassium phosphate buffer, pH 7.4, for peroxynitrite. All reactions were carried out in a final reaction volume of 1 mL. Under these assay conditions, oxidation of KMBA is comparable during the time course of the control

reaction [16,17]; thus, the relative efficiency of cellular antioxidants towards various oxidants is compared under quantitatively similar prooxidant challenge. Ethylene formation in control and sample reactions was analysed at 15 min intervals by gas-chromatographic analyses of 0.5 mL aliquots removed by syringe from the head space of the reaction vessel [16,17] with a Varian model 3400 gas chromatograph (Varian, Palo Alto, CA) and the TOSC values quantified from the equation: TOSC $= 100 - (100 \times \int \mathrm{SA}/\int \mathrm{CA})$, where $\int$ SA and $\int$ CA are the integrated areas calculated under the kinetic curve produced during the reaction course for respective sample (SA) and control (CA) reactions [16]. An experimental TOSC will, respectively, indicate no inhibition of ethylene formation ($\int \mathrm{SA}/\int \mathrm{CA} = 1$) and maximum scavenging capacity with no KMBA oxidation (SA $= 0$). The specific TOSC (TOSC per 1 μg of protein) was calculated by dividing the experimental TOSC values by the μg of protein obtained from linear plots of TOSC vs. μg/mL contained in the assay (see Results section for detailed explanation). Protein concentration was determined with a Pierce BCA™ Protein kit with bovine serum albumin used as standard.

3. Results

Figure 1 shows typical inhibition curves (decreasing slopes) caused by *Hexagenia* nymphal S9 of ethylene production from peroxyl radical-dependent (from thermal decomposition of ABAP) oxidation of KMBA. Similar inhibition curves by *Hexagenia* nymphal S9 fractions were obtained for ROS generated from the Fenton (hydroxyl radicals) and SIN-1 (peroxynitrite) reactions (not shown). After obtaining the inhibition curves for the three oxidant generating systems used, the

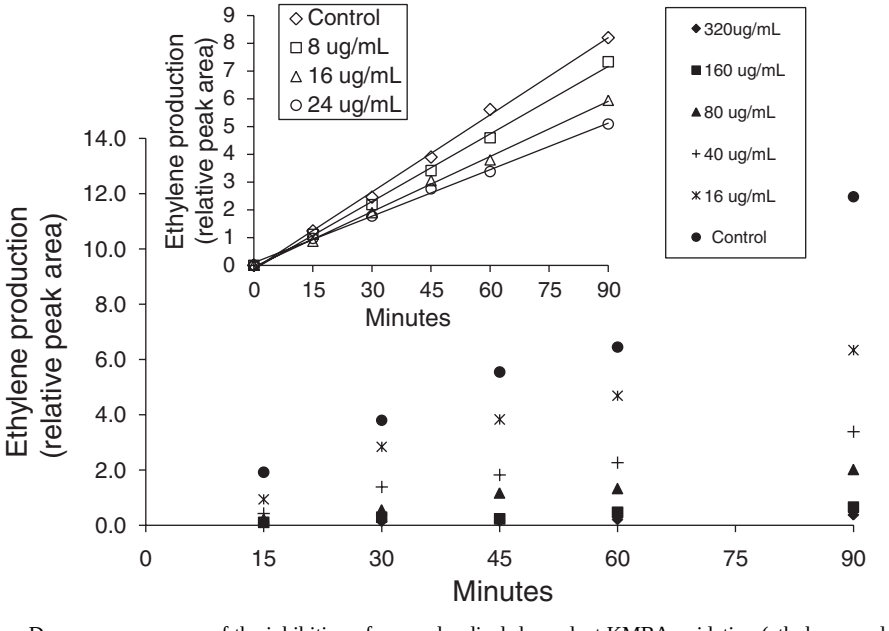

Figure 1. Dose response curve of the inhibition of peroxyl radical-dependent KMBA oxidation (ethylene production) caused by increasing concentrations of *Hexagenia* nymphal S9 protein. The time course is shown over 90 minutes. The range of protein concentrations was from 16–320 μg/mL. After such inhibition curves are generated they were refined to produce the linear range of TOSC vs. S9 protein concentration (see Materials and methods section) as shown in the inset to Figure 1 (the linear range is between 8–24 μg/mL).

curves are refined (Figure 1 inset; shown for peroxyl radical only) to produce a range of linear integrated areas under the curves for each oxidant (Figure 2). These linear graphs are relative to the control curves and are presented as TOSC values versus S9 protein concentrations in the reaction mixture.

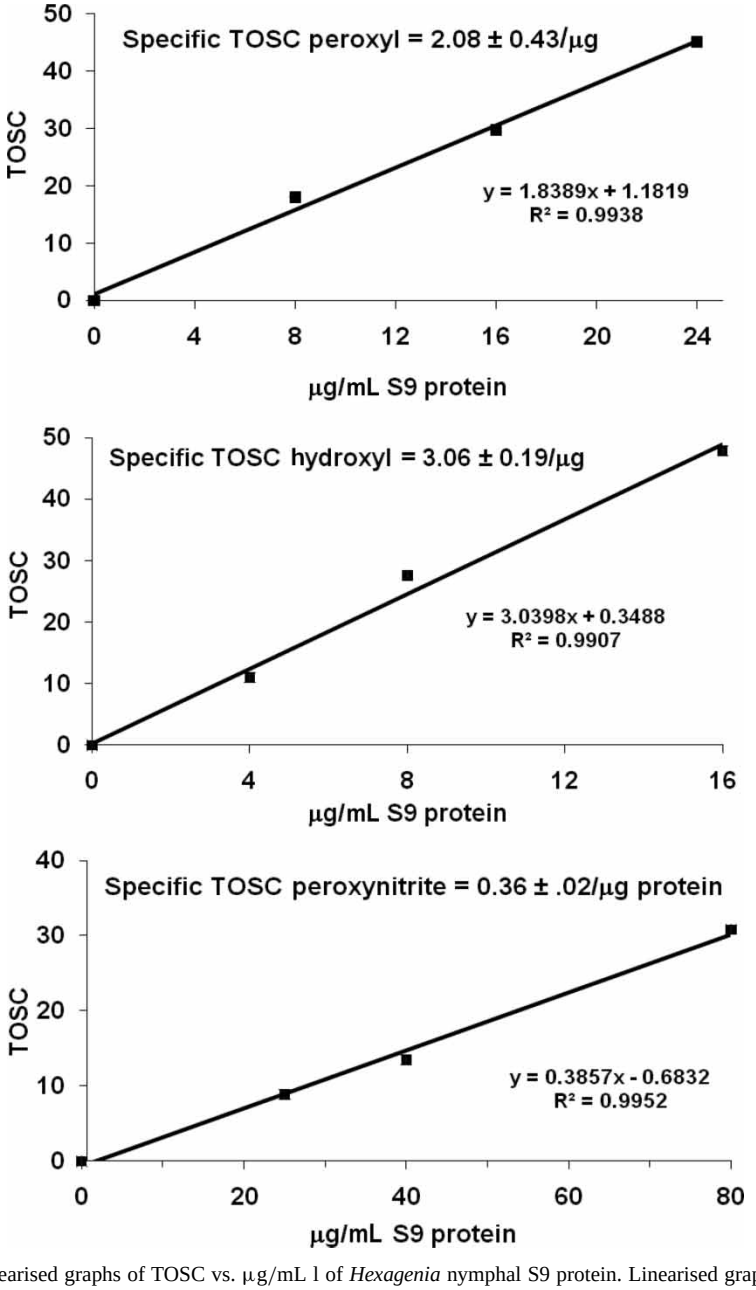

Figure 2. Linearised graphs of TOSC vs. µg/mL l of *Hexagenia* nymphal S9 protein. Linearised graphs from top to bottom, respectively, are TOSC values for peroxyl radical- hydroxyl radical- and peroxynitrite as a function of S9 protein. Specific TOSC (TOSC/µg *Hexagenia* nymphal S9 protein) is determined by interpolation of the linearised graphs (see explanation of Figure 2 derivation in the Results section) and are shown in each of the graphs ± SD of three to four determinations.

When the areas under the inhibition curves are refined to show the linear range of inhibition by *Hexagenia* nymphal S9, a specific TOSC (TOSC/μg S9 protein ± SD) for each of the oxidants generated can be determined by interpolation of these linear relationships. Figure 2 compares the specific TOSC values (interpolated from the linear TOSC vs. concentration curves) by *Hexagenia* nymphal S9 fractions. The specific TOSC values (±SD, $n = 3$) per μg protein are 2.08 ± 0.43 for peroxyl radicals (top), 3.06 ± 0.19 for hydroxyl radicals (middle) and 0.36 ± 0.02 for peroxynitrite (bottom). The kinetic curves in the inset to Figure 1 were done accordingly. After noting that the areas under the curves of the main graph were not linear, time courses were determined again for ethylene from KMBA production for the lowest point (16 μg/mL) of the main graph, a point between 16 μg/mL and 40 μg/mL (24 μg/mL) and a point below 16 μg/mL (8 μg/mL). The integrated areas under these ethylene production curves were linear as shown for peroxyl radical in Figure 2. In the exact same manner we determined the linear range of TOSC vs. protein concentration for the other oxidants (peroxynitrite and hydroxyl radical). The SD values shown in Figure 2 reflect between three and four replications depending on the oxidant employed to generate the various TOSC vs. concentration curves.

Figure 3 compares the specific TOSC of *Hexagenia* nymphal S9 for peroxyl radicals with that of Trolox (a water soluble vitamin E analogue), reduced glutathione (GSH) and rat liver cytosol to relate the data in terms of Trolox and GSH TOSC equivalents and for comparative purposes against the mammalian system. The specific TOSC values for Trolox (0.023 per μM), GSH (0.84 per μM) and rat liver cytosol (0.40 per μg) are from [16] from the linear range of TOSC vs. antioxidant curves. The μM values were converted to μg for comparison with the μg equivalents of the biological tissues. *Hexagenia* nymphal S9 contained 11.9% of Trolox equivalents and 77.0% of GSH equivalents on a per μg basis. These data indicate that both α-tocopherol and GSH may be important factors reflecting the scavenging capacity toward peroxyl radical. This is not to say that these are definitely the only scavengers involved, however, we do know that there is both a selenium-dependent and independent glutathione peroxidase activity in *Hexagenia* S9 as well as glutathione S-Transferase. *Hexagenia* nymphal S9 showed 20% greater peroxyl radical scavenging capacity than did rat liver cytosol.

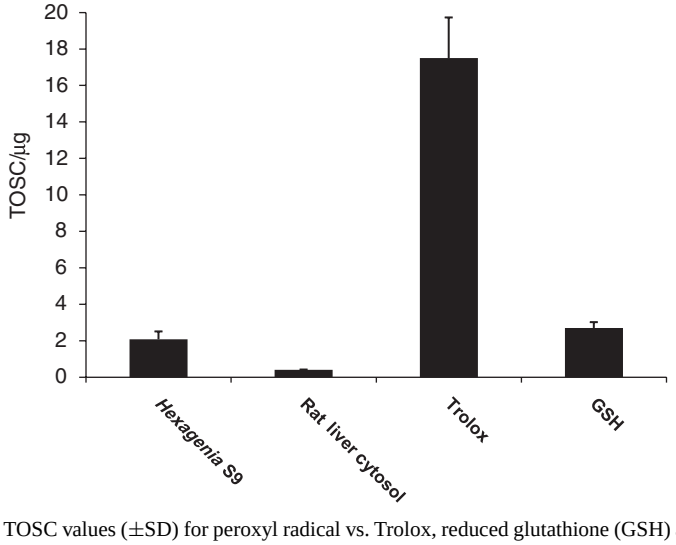

Figure 3. Specific TOSC values (±SD) for peroxyl radical vs. Trolox, reduced glutathione (GSH) and rat liver cytosol. The values for the these are from [16]. *Hexagenia* nymphal S9 is, respectively, 11.9% and 77% of Trolox and GSH equivalents. Values for *Hexagenia* S9 is from the current manuscript.

4. Discussion

The capacity to scavenge or neutralise ROS is an important part of an organism's defences against oxidative damage that may be caused by these oxidants. The TOSC assay reflects the full capability of neutralising specific forms of ROS, such as peroxyl radicals, hydroxyl radicals and peroxynitrite [17]. The measurement of individual low molecular weight antioxidants or antioxidant enzymes can be sensitive in revealing a pro-oxidant stressor, which is useful in understanding certain mechanisms and responses to these stressors, but often fails to provide or allow summarisation as a single metric or index [6,16,17]. On the other hand, TOSC is sometimes less sensitive, but of greater overall significance, because a reduced capability to neutralise ROS can indicate health impairment, providing an important indication regarding the involvement of specific ROS or their metabolic pathways in pollutant-mediated oxidative stress [9].

The specific TOSC values (TOSC/μg protein) for the S9 fractions of *Hexagenia* nymphal S9 toward peroxyl and hydroxyl radicals and peroxynitrite presented under Results (Figure 2) indicate a capability for scavenging or neutralising the three ROS studied in the present manuscript. The specific TOSC for hydroxyl radical by the *Hexagenia* S9 fraction was greater than for the other two oxidants; the specific TOSC order was hydroxyl radical > peroxyl radical ≫ peroxynitrite. The relatively high hydroxyl and peroxyl radical specific TOSC of *Hexagenia* nymphal S9 is most commonly associated with oxidative damage *in vivo*. As mentioned in the Introduction to this paper numerous pathways result in hydroxyl radical production (electron transport, various enzymes). Peroxyl radical production arises from its propagation in the chain of radicals generated through lipid peroxidation, until a chain-breaking antioxidant can be encountered (e.g. α-tocopherol). Therefore, we compared and contrasted *Hexagenia* nymphal S9 specific TOSC peroxyl radical in terms of its equivalent scavenging capacity to that of rat liver cytosol, Trolox® and reduced glutathione (GSH). Both Trolox® and GSH have been shown to be better scavengers of peroxyl radical than of hydroxyl radical or peroxynitrite [17]. This comparison of *Hexagenia* nymphal S9 TOSC allows the S9 specific TOSC to be expressed in Trolox® and GSH specific TOSC equivalents, which are 11.9% and 77%, respectively on a per μg basis.

The Trolox® equivalent scavenging capacity for peroxyl radical of about 11.9% that of Trolox® suggests that α-tocopherol or scavengers with the scavenging power α-tocopherol may be in relatively effective abundance in *Hexagenia* nymphal S9 fractions. Lipid-soluble antioxidants such as tocopherols, and carotenoids have not been well studied in insects [21] but may have important antioxidant roles. Based on an extensive literature review, there are no data on the levels of lipid-soluble antioxidants in *Hexagenia* spp. Tocopherols and carotenoids are found in freshwater algae, including *Pseudokirchneriella subcapitata*, *Chlorella vulgaris*, *Cladophora glomerata* and *Enteromorpha ahlneriana* [22,23] and these antioxidants protected these algae from oxidative stress. As stated earlier, algae, bacteria and detritus constitute most of the *Hexagenia* nymph diet and likely provide some antioxidant protection to *Hexagenia* from tocopherols and/or carotenoids. Unfortunately, we were not able to assess actual levels of these or other antioxidants in the *Hexagenia* samples that we studied. Nevertheless, the present findings certainly indicate that measurement of antioxidant levels in *Hexagenia* fractions would be of heuristic value.

When the data were expressed as reduced glutathione (GSH) equivalents, TOSC for the S9 fraction was about 77% that of GSH. That *Hexagenia* nymphal S9 fractions could garner antioxidant protection from GSH was not particularly surprising in light of subsequent studies by our laboratory, which revealed that *Hexagenia* nymphal S9 displayed glutathione S-transferase, glutathione peroxidase and glutathione reductase activities [24]. Moreover, a substantial literature indicates the presence of both oxidised (GSSG) and reduced glutathione and glutathione utilising enzymes in various insect tissues [25–27].

Hexagenia nymphal S9 was 20% greater in potency as a peroxyl radical scavenger than was that of rat liver cytosol. The S9 fraction is a mixture of both microsomes and cytosol; thus, the

difference in scavenging strength may have been owing to scavenging by microsomes, which as a membrane fraction should contain a greater complement of lipid-soluble antioxidants, likely tocopherols and/or carotenoids. Also, the cytosolic portion of the *Hexagenia* S9 may contain somewhat more potent scavenging power than the rat liver cytosol, which only further analysis would be able to discern.

It is important to note that TOSC does not account for antioxidant enzymes, e.g. SOD, catalase, and glutathinone peroxidase in the holistic framework of protection against peroxyl radical and peroxynitrite [17]. With these ROS, TOSC reflects principally low molecular weight (LMW) scavengers, e.g. vitamin E, GSH, etc. This is clear in evaluating the peroxyl radical scavenging capacity as Trolox® and GSH equivalents. In the hydroxyl radical system, in which the iron plus ascorbate system is used, it is possible that the observed TOSC could reflect enzymatic removal of H_2O_2 by either adventitious catalase or glutathione peroxidase. This putative removal H_2O_2 removes the precursor of the hydroxyl radical and thus, can give the appearance of scavenging capacity. We recommend that inhibitors of catalase, e.g. azide, and glutathione peroxidase, e.g. misonidazole, be included in the evaluation of hydroxyl radical scavenging capacity by the TOSC assay. Thus, holistic understanding of a tissue's capacity to defend against oxidative stress requires assessment of all antioxidant enzymes and LMW scavengers; TOSC measures only the amount of ROS that a tissue can scavenge [16,17]. The data do illustrate, however, that the same antioxidants present in *Hexagenia* nymphal S9 can react differentially against different oxidants, a fact that is often overlooked when evaluating overall antioxidant capacity of organisms.

Of particular interest is the relatively much lower specific TOSC value for peroxynitrite as compared to hydroxyl and peroxyl radical TOSC values. This may reflect a low capacity of mayfly nymphs to produce peroxynitrite via NO synthase. NO synthase has been reported to be involved in adult cockroach nervous systems [28] but to the best of our knowledge there is no data to indicate this in sediment dwelling nymphal stages of aquatic insects.

TOSC has been used as a biomarker of pollutant-mediated oxidative stress in various freshwater and marine organisms [6,10,29–32]. Our aim is to develop and characterise *Hexagenia* nymphs for use as sentinels of Lake Erie sediment habitat, especially the western basin of this lake where there are substantial contaminant loadings [33–35]. *Hexagenia* is already a recognised bioindicator of Lake Erie health and that of numerous other habitats worldwide. *Hexagenia* nymphs reappeared in the sediments of western Lake Erie in the early 1990s after being almost nonexistent for nearly 40 years [36]. This reemergence was thought to reflect the success of pollution-abatement programs in combination with the appearance of invasive zebra mussels in 1986 that perturbed the trophic balance of Lake Erie's nearshore habitat. Although information prior to the 1950s is limited, it is known that the disappearance of *Hexagenia* from Lake Erie was preceded by a period of anoxia or extreme hypoxia [37], which was ascribed to organic loadings from municipal wastes. Between 1960 and 1990, few mayflies were found in Lake Erie [38].

Certain biological reference points or category descriptors, e.g. nymph density ranges, were recently established based on the historic abundances of *Hexagenia* spp. in Lake Erie's western basin [39]. A part of the rationale for these reference points was to facilitate communication to agencies regarding progress toward the goals of lake-wide management plans [38]. Biochemical reference points would add important criteria to environmental management strategies. Our specific rationale is to employ the nymphal stages of *Hexagenia* as early warning indicators of ensuing deleterious health by understanding well established biochemical changes that can be used as biochemical biomarkers. *Hexagenia* nymphs may prove to be ideal sentinel organisms because they spend all but the last several hours of their lifespan burrowed within the sediments of Lake Erie [40] and hence are exposed to numerous classes of contaminants for as long as two years. Furthermore, parts of Lake Erie are characterised in summer by occasional rapid shifts between normoxic and severely hypoxic (sometimes nearly anoxic) states [40]. A consequence of such transitions can be reperfusion injury upon reoxygenation following periods of extreme

hypoxia [42,43]. Reperfusion injury is well known to perturb the antioxidant defence mechanisms of organisms, including insects. The significant potential for reperfusion injury in the Lake Erie setting is yet another cogent reason to assess baseline TOSC values if one is to study the alterations in antioxidative defences resulting from these oxygen fluctuations. Finally, before these organisms can be used efficaciously as sentinel organisms at the molecular level, it is essential to characterise biomarker responses in terms of their first principles, i.e. their existence and basal levels as well as their equivalent potency to classical purified antioxidants.

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