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Carotenoid glycosides from cyanobacteria are teratogenic in the zebrafish (*Danio rerio*) embryo model

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Abstract

Toxicity of cyanobacteria is widely associated with production of several well-described toxins that pose recognized threats to human and ecosystem health as part of both freshwater eutrophication, and episodic blooms in freshwater and coastal habitats. However, a preponderance of evidence indicates contribution of additional bioactive, and potentially toxic, metabolites. In the present study, the zebrafish (*Danio rerio*) embryo was used as a model of vertebrate development to identify, and subsequently isolate and characterize, teratogenic metabolites from two representative strains of *C. raciborskii*. Using this approach, three chemically related carotenoids - and specifically the xanthophyll glycosides, myxol 2'-glycoside (**1**), 4-ketomyxol 2'-glycoside (**2**) and 4-hydroxymyxol 2'-glycoside (**3**) - which are, otherwise, well known pigment molecules from cyanobacteria were isolated as potentially teratogenic compounds. Carotenoids are recognized "pro-retinoids" with retinoic acid, as a metabolic product of the oxidative cleavage of carotenoids, established as both key mediator of embryo development and, consequently, a potent teratogen. Accordingly, a comparative toxicological study of chemically diverse carotenoids, as well as apocarotenoids and retinoids, was undertaken. Based on this, a working model of the developmental toxicity of carotenoids as pro-retinoids is proposed, and the teratogenicity of these widespread metabolites is discussed in relation to possible impacts on aquatic vertebrate populations.

GRAPHICAL ABSTRACT

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Keywords

Cyanobacteria; Carotenoid; Myxoxanthophyll; Zebrafish; Teratogenicity; Retinoid

1. Introduction

Cyanobacteria are ubiquitous photosynthetic prokaryotes that, as the “blue-green algae,” are perhaps most conspicuous in the environment in association with episodic and seasonal algal blooms, as well as eutrophication of freshwater and coastal marine habitats. A growing number of cyanobacterial species are recognized producers of toxic metabolites linked to various human health concerns, including acute poisoning and chronic health effects (e.g. cancer, neurodegenerative disease), particularly as part of so-called “harmful algal blooms” (HABs) (Carmichael, 2008). In addition, a considerable body of knowledge suggests that cyanobacterial toxins may impact aquatic animal health (e.g. fish kills, poisoning of wildlife) alongside broader effects on aquatic ecosystems including declines and/or compositional shifts in both invertebrate and vertebrate populations (Leão et al., 2009; Zanchett and Oliveira-Filho, 2013).

Despite a recognized diversity of bioactive compounds from cyanobacteria, studies of cyanobacterial toxicity have primarily focused on a relatively small number of metabolites including several water soluble alkaloids and peptides linked to human intoxications as hepatotoxins (i.e. microcystins, cylindrospermopsin) and neurotoxins (e.g. anatoxin-a, saxitoxin). However, numerous studies, specifically comparing crude extracts to pure toxins, have consistently demonstrated contributions of additional, and seemingly unrelated, bioactive metabolites to the toxic potential of cyanobacteria (e.g. Saker et al., 2003; Nogueira et al., 2006; Berry et al., 2009; Acs et al., 2013; Jonas et al., 2014). These additional toxic metabolites have remained, in most cases, uncharacterized both chemically and toxicologically.

In previous studies, the zebrafish (*Danio rerio*) embryo was employed as a model of vertebrate development to evaluate teratogenicity (i.e., developmental toxicity) of metabolites from cyanobacteria (e.g. Berry et al., 2007; Berry et al., 2009; Jaja-Chimedza et al., 2012, 2015; Walton et al., 2014). Notable among these, *Cylindrospermopsis raciborskii*, a species frequently (and increasingly) linked to freshwater HABs, was found to produce teratogenic metabolites (Berry et al., 2009). With respect to toxic potential of the species, *C. raciborskii* has been most commonly associated with production of toxic alkaloids, namely

cylindrospermopsin (CYN) and saxitoxin (STX), which have been well characterized as hepatotoxic and neurotoxic compounds, respectively (Kinnear, 2010; Wiese et al., 2010). Evaluation in the zebrafish embryo system clearly demonstrated that observed teratogenicity, however, was unrelated to these recognized toxins, and suggested a contribution of otherwise uncharacterized lipophilic metabolites from the species (Berry et al., 2009).

In the present study, the zebrafish embryo teratogenicity assay was employed to isolate (via bioassay-guided fractionation) and subsequently characterize additional bioactive metabolites from *C. raciborskii*, and specifically identified a series of carotenoid glycosides (Fig. 1) as apparently potent teratogens. Indeed, carotenoids including, in particular, a few notable congeners (e.g., β -carotene) are well known “pro-retinoids,” serving as metabolic precursors to retinoic acid (RA) and related retinoids that, in turn, are recognized to be potent teratogens. The observed teratogenicity of the cyanobacterial carotenoid glycosides, therefore, would be consistent with a pro-retinoid mechanism of developmental toxicity. In order, to evaluate whether the observed teratogenicity could be extended to other carotenoids, several chemically diverse carotenoids (including recognized pro-retinoids), as well as apocarotenoids and retinoids, were subsequently evaluated in the zebrafish embryo model. A working model of the teratogenicity of the carotenoids is accordingly proposed.

2. Methods and materials

2.1. *C. raciborskii* culture material

Carotenoids in the present study were isolated from cultured material of two strains of *C. raciborskii*. Cultures of *C. raciborskii* AQS were provided by Centro de Investigação Marinhae Ambiental (Laboratory of Ecotoxicology) at the University of Porto (Portugal), and originally isolated from aquaculture ponds in Australia (Saker and Eaglesham, 1999). Cultures of *C. raciborskii* 121-1 were derived from samples isolated from Lake Catemaco (Veracruz, Mexico; Berry et al., 2012). Cultures of both were maintained as previously described (Berry et al., 2007, 2009; Gantar et al., 2008). Algal cells/biomass were harvested after three to four weeks by centrifugation, and subsequently freeze-dried for extraction.

2.2. Zebrafish embryo teratogenicity assay

The zebrafish (*Danio rerio*) embryo was used as a model of vertebrate development to evaluate teratogenicity, and toxicity more generally. Maintenance and breeding of adult zebrafish, as well as the zebrafish embryo teratogenicity assay, was done as previously described (Berry et al., 2007, 2009; Jaja-Chimedza et al., 2012; Walton et al., 2014). Assays were conducted in polypropylene 24-well plates (Evergreen Scientific, Los Angeles, CA) with five embryos (4- to 32-cell stage) per well, i.e., replicate ($n = 3$), in E3 medium (Brand et al., 2002). Embryos were observed at 1, 2, 3 and 5 days post fertilization (dpf) with a dissecting light microscope to assess mortality and developmental toxicity. Mortality was scored by two complementary criteria including: coagulation of embryos at early stages (2 dpf), and deformity sufficiently severe (e.g., lack of somites, lack of heartbeat) to be considered effectively lethal. Lethal and effective concentrations for 50% mortality and deformity were estimated, respectively, as LC_{50} and EC_{50} by probit analysis. Following the identification of **1–3** as carotenoids (see below), assays were subsequently conducted in dim

light with incubation of assay plates (0–5 dpf) in the dark, and at lowered temperatures (25 °C versus optimal 28 °C), to minimize potential degradation of the temperature sensitive and photolabile carotenoids. All breeding and bioassays involving zebrafish were conducted under protocols approved by the FIU Institutional Animal Care and Use Committee (IACUC), and performed by trained investigators.

2.3. Extraction and purification of carotenoids by bioassay-guided fractionation

The zebrafish embryo toxicity assay was used to guide fractionation toward purification of teratogenic metabolites from both strains of *C. raciborskii*. With no prior knowledge of their chemical identity, carotenoids were purified from the two strains independently, and specifically using bioactivity, i.e., developmental toxicity, to guide isolation. Purification from the two strains, therefore, followed two separate isolation schemes. In both cases, freeze-dried biomass was twice extracted in chloroform (10 mg biomass/1 mL of extraction solvent), and evaluated for teratogenicity (i.e. embryo deformities, developmental dysfunction) at several dilutions; the observed teratogenicity was employed at each subsequent fractionation step to identify relevant bioactive fractions. Extracts were subsequently fractionated by normal phase (silica gel, 60 Å, Commercial 40–63 µm) flash column chromatography using two separately developed methods, and specifically either a step-wise gradient of (1) ethyl acetate in hexane, followed by 10% methanol in ethyl acetate, for *C. raciborskii* AQS; or (2) acetone in hexane, followed by 100% methanol, for *C. raciborskii* 121-1. The most polar (10% methanol in ethyl acetate) fraction from AQS was found to be bioactive, and fractionated further (following concentration *in vacuo*) using reverse-phase (C-18) solid-phase extraction (SPE, Extract-Clean™ C18-HC 10 g/75 mL) with stepwise gradient of elution by methanol in water. The resulting bioactive fraction (95% methanol in water) was separated by high-performance liquid chromatography (HPLC), coupled to photodiode array (PDA) detector. Isocratic elution (65:35 acetonitrile/water) and a reverse-phase column (Phenomenex Luna C5, 5 µm, 100 Å, 100 mm × 4.6 mm) enabled purification of two bioactive compounds (**1** and **2**), specifically eluting at 6.7 and 8.6 min. Similarly, the most polar fraction (100% methanol eluate) from 121-1 was found to be bioactive, and subsequently separated by HPLC (identically as AQS) affording a single bioactive peak (**3**) eluting at 4.6 min. Following the purification of initial quantities of the compounds, and tentative identification as carotenoids, subsequent purification was done in dim yellow light, and all samples were stored in the dark (at –20 °C), to minimize degradation of these light- and heat-sensitive compounds.

2.4. Chemical characterization of isolated carotenoids

The bioactive compounds purified from AQS were chemically characterized by UV/Vis spectroscopy, electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR) spectroscopy. Due to instability of the compounds (see below), UV/Vis absorbance and MS spectra were primarily obtained with an online PDA detector and mass spectrometer, respectively, coupled to HPLC. Chemical characterization based on UV/Vis spectroscopy specifically included evaluation of the maximal absorbance (λ_{max}) within “three-peak spectra” indicative of carotenoids, and associated *spectral fine structure*. The latter specifically included calculation of the relative peak height of the second and third highest λ_{max} within this spectrum (i.e. %III/II = peak height of III/peak height of II × 100%)

that has been extensively used to determine the structure of carotenoids (Takaichi and Shimada, 1992). Low-resolution ESI-MS analysis was done using a Thermo TSQ Quantum Access electrospray ionization (ESI)/triple quadrupole instrument coupled to a Thermo Accela UHPLC. High-resolution ESI-MS (HRESIMS) was achieved using a Thermo Q Exactive Orbitrap mass spectrometer, similarly coupled to an Accela UHPLC systems. Chemical characterization by NMR included one-dimensional (i.e., ^1H), and two-dimensional experiments (i.e., homonuclear COSY) on a Bruker AVANCE 400 MHz instrument.

In addition to spectroscopic characterization, purified compounds and bioactive fractions were evaluated, based on the tentative identification of the bioactive metabolites as carotenoids, using the previously described (Louda, 2008) analytical method specific for algal pigments including carotenoids. Samples were spiked with relevant carotenoid standards (i.e., myxoxanthophyll and aphanizophyll; DHI, Denmark) for confirmatory identification.

Myxol 2'-glycoside (1)—red-orange amorphous solid; UV/Vis (PDA) λ_{max} 295, 368, 450, 478, and 509 nm, %III/II = 58; ESI-MS m/z 567.4 (loss of methylpentoside), 730.5 (M^+), 753.5 ($[\text{M}+\text{Na}]^+$); HRESIMS m/z 730.4801 ($\text{C}_{46}\text{H}_{66}\text{O}_7$, $[\text{M}]^+$, mmu of -2.24), ring-and-double-bond equivalents (RDB) = 14.

4-Ketomyxol 2'-glycoside (2)—red-orange amorphous solid; UV/Vis (PDA) λ_{max} 321, 449sh, 480 and 509 nm, %III/II = 12; ESI-MS m/z 581.4 (loss of methylpentoside), 744.4 (M^+), 767.4 ($[\text{M}+\text{Na}]^+$). HRESIMS m/z 744.4548 ($\text{C}_{46}\text{H}_{64}\text{O}_8$, $[\text{M}]^+$, mmu of -2.24), RDB = 15.

4-Hydroxymyxol 2'-glycoside (3)—UV/Vis (PDA) λ_{max} 451, 476, and 507 nm, %III/II = 56; ESI-MS m/z 583.4 (loss of methylpentoside), 746.5 (M^+), 769.5 ($[\text{M}+\text{Na}]^+$); HRESIMS m/z 746.4762 ($\text{C}_{46}\text{H}_{66}\text{O}_8$, $[\text{M}]^+$, mmu of 0.850), RDB = 14.0.

2.5. Comparative toxicology of carotenoids and retinoids

To determine whether the observed teratogenicity was limited to the xanthophyll glycosides isolated from *C. raciborskii*, or rather common to other members of this chemical family, and to compare to the teratogenicity of retinoids, several chemically distinct (Fig. 2) carotenoids including carotenes, xanthophylls and apocarotenoids, along with several retinoids, were evaluated in the zebrafish embryo teratogenicity assay. Commercially available compounds tested, in this regard, included: lutein (Cayman Chemical, Ann Arbor, MI, U.S.A.); canthaxanthin and zeaxanthin (Fluka, Switzerland); β -carotene, trans- β -apo-8'-carotenal, *all-trans* retinoic acid, *all-trans* retinaldehyde and retinol (Sigma-Aldrich, St. Louis, MO, U.S.A.). Each compound was tested in the zebrafish teratogenicity assay, alongside **1** and **2**, and relevant solvent (i.e. methanol) only and untreated controls, in triplicate at 60, 30, 15, 5 and 1 μM as described (see 2.2. *Zebrafish embryo teratogenicity assay*). Toxicity was assessed based on both mortality and observed developmental deformity; mortality was determined using two complementary criteria (i.e., coagulation and severe deformity; see 2.2. *Zebrafish embryo teratogenicity assay*). To assess relative toxicity,

the percent mortality/developmental deformity (i.e., number of deformed embryos/number of live embryos $\times$ 100%) for each treatment was calculated. Percent mortality/deformity at each concentration (1, 5, 15, 30 and 60 μ M) and observational time point (1, 2, 3, 4 and 5 dpf) were compared by Fisher Exact Test using Analystsoft software (Walnut, CA, U.S.A.) to determine statistical significance relative to solvent-only (i.e., methanol) controls. Embryos, including representatives of development deformities, and relevant controls (i.e., untreated normal embryos), were photographed at each test concentration (1–60 μ M) and observational time-point (1–5 dpf).

3. Results

3.1. Isolation and chemical characterization of teratogenic carotenoids

Chloroform extracts, and subsequent fractions, from both *C. raciborskii* isolates (AQS and 121-1) were evaluated by the zebrafish embryo assay, and found to be teratogenic. For AQS, a consistent pattern of teratogenicity was observed at each sequential fractionation step (i.e. flash column chromatography, C-18 SPE) enabling the purification of two bioactive components (**1** and **2**) in adequate quantities for subsequent chemical characterization. The development defects of embryos exposed to these extracts and subsequent fractions was characterized by a general lack of differentiation of head and tail, and associated features (e.g., eye), as well as anterior aggregation (suggesting inhibited migration) of melanophores. Developmental defects were generally severe enough in all cases, including subsequent bioassay-guided fractionation, to be effectively lethal to embryos. In parallel, qualitatively similar teratogenicity was observed for extracts, and subsequent fractions, from *C. raciborskii* 121-1, and used to identify a single bioactive component (**3**). Compound **3** was not purified in sufficient quantity for extensive spectroscopic analysis, but chemical characterization based on UV/Vis spectroscopy (Fig. 3) and mass spectrometry, specifically coupled to analytical separation by HPLC, as well as a previously validated analytical technique for algal pigments (Louda, 2008), was sufficient for confirmatory identification of this compound.

Initial chemical characterization of purified **1** and **2** included UV/Vis spectroscopy, ESI-MS and NMR. Both compounds were found to be highly unstable as indicated by, for example, loss of red-orange color of the compounds following mild heating (to evaporate solvents during isolation), and upon standing at room temperature and ambient light. Likewise, all attempts to conduct NMR spectroscopy with the purified compounds indicated clear degradation of the compounds such that these analyses were not possible. This instability was, in fact, consistent with tentative identification of the compounds as carotenoids. Chemical characterization of carotenoids generally relies on, as the minimal criteria for acceptable identification, UV/Vis spectroscopy in conjunction with mass spectrometry, and the use of appropriate reference standards (Britton et al., 2004). The utility of NMR analyses (without derivatization) is largely limited by this instability, and consequent degradation. In particular, the so-called “three-peak spectra” of carotenoids - the maximal peaks (I, II and III) of UV/Vis absorbances (λ_{max}) between 420 and 520 nm (e.g., Fig. 3) - is highly indicative of structure, and particularly used to determine conjugation and cyclization of the variants. Within this, the *spectral fine structure*, and specifically relative peak heights

(i.e., %III/II), is capable of determining conjugation and cyclization within the carotenoid structure (Takaichi and Shimada, 1992). In conjunction with UV/Vis spectroscopy, HRESIMS enabled generation of molecular formulae, and consequently assignment of carotenoid structures.

Based on these analyses, **1** was tentatively identified as the previously described xanthophyll, myxol 2'-glycoside (Fig. 1). The three-peak spectrum of **1** (450, 478 and 509 nm), and corresponding fine structure (%III/II = 58%), generally enabled tentative assignment as a β -carotenoid, and specifically a xanthophyll, typical of those reported for cyanobacteria. Mass spectrometric analysis of **1** identified a molecular mass of 730.4801, and corresponding molecular formula of $C_{46}H_{66}O_7$. Notably, although ESI-MS was employed, the ionized molecular ion (i.e., M^+) rather than the expected protonated molecular ion (i.e., $M+H^+$) was observed. However, the presence of the stable molecular ion in ESI-MS is, in fact, consistent with previous observations for carotenoids, and has been specifically linked to the high degree of conjugation, and the resulting ability to withstand loss of an electron, among these neutral analytes (Guaratini et al., 2005). The molecular formula determined by HRESIMS, and specifically the apparent presence of oxygen, is further suggestive of a xanthophyll (i.e., oxygen-containing carotenoid). Moreover, fragmentation patterns in the MS analysis, and specifically the observed loss of 163 amu (i.e. m/z 567.4), is indicative of the presence of a sugar moiety, and particularly a methylpentoside, typical of many of the xanthophyll glycoside produced by cyanobacteria (Takaichi et al., 2001, 2005; Mohamed et al., 2005). Although the sugar moiety of **1** was not characterized in the present study, it has been most typically associated with the methylpentose, fucose (Takaichi et al., 2001, 2005).

Similar analyses enabled identification of **2** as the congener, ketomyxol 2'-glycoside. The three-peak spectrum of **2** (449, 480 and 509 nm) and spectral fine structure, and specifically the small %III/II (12%) of the associated fine structure, was consistent with a carotenoid containing a carbonyl within the β -ionone ring (effectively extending the conjugation within the chromophore). The molecular formula ($C_{46}H_{64}O_8$) assigned, based on the molecular ion (m/z 744.4548) identified in HRESIMS analysis, as well as fragmentation patterns which specifically included, similar to **1**, apparent loss of a methylpentoside (163 amu, m/z 581.4), likewise, were highly consistent with the proposed assignment as the 4-keto congener of the myxol glycoside (i.e., "keto-myxoxanthophyll").

To confirm the identity of **1** and **2**, samples of the purified compounds were evaluated by a previously validated (Louda, 2008) technique for analysis of algal pigments (i.e. chlorophyll, carotenoids). This ternary HPLC-based method is an effectively confirmatory technique capable (based on relative retention time, UV/Vis absorbance and use of relevant analytical standards) of unambiguous identification of carotenoids and other pigments (Louda, 2008; Hagerthy et al., 2006). Accordingly, the identity of both **1** and **2** were confirmed. This confirmatory technique was additionally applied to bioactive fractions from *C. raciborskii* 121-1 (see above), and specifically enabled identification of the single bioactive component (**3**) from this strain as the previously described 4-hydroxymyxol 2'-glycoside (or "aphanizophyll").

3.2. Teratogenicity of retinoids in the zebrafish embryo model

Based on the proposed pro-retinoid mechanism of teratogenicity of **1–3**, three primary retinoids, RA, retinal and retinol, were assessed for developmental toxicity. All retinoids evaluated were teratogenic and effectively lethal by 2 dpf with a relative potencies of RA > retinal > retinol (Fig. 4). In the present study, RA was tested at concentrations well above the LC₅₀ and EC₅₀ (for teratogenicity) previously reported in the zebrafish embryo which are generally in the nanomolar to sub-nanomolar range, respectively (Hermann, 1995; Selderslaghs et al., 2009; Wang et al., 2014). And, indeed, teratogenicity was effectively lethal (Fig. 5A–B) at even the lowest concentration of RA tested (1 µM) which is several orders of magnitude above previously reported LC₅₀/EC₅₀ values. Retinal and retinol, on the other hand, were quantitatively less toxic than RA. Both resulted in developmental deformities which were sufficiently severe to be lethal (and as such deformity was equivalent to mortality). However, the corresponding EC₅₀/LC₅₀ of retinal and retinol were lower than RA, and specifically calculated as 1.8 ± 0.7 µM and 9.2 ± 7.6 µM, respectively.

3.3. Teratogenicity of carotenoids in the zebrafish embryo model

A comparative toxicology study of several carotenoids in the zebrafish embryo teratogenicity assay was conducted to assess whether teratogenicity could be extended to other members of this widespread chemical family. Aside from teratogenicity of the carotenoids, however, development was generally delayed (see Fig. 5) in the exposure studies with carotenoids and retinoids (see previous section), specifically compared to standardized staging series (Kimmel et al., 1995; OECD, 2013), for all embryos including untreated controls. Under optimal conditions zebrafish embryos typically hatch at approximately 3 dpf, but embryos in the exposure studies remained unhatched through the 5 days of the study. Embryos were clearly underdeveloped (Fig. 5) at all stages with respect to organogenesis and other aspects when compared to typical studies. For example, in the present study embryos incubated for 3 dpf were equivalent in developmental stage to 31 hpf embryos typically reported in the literature, while 5 dpf embryos were equivalent to 3 dpf (Kimmel et al., 1995).

Delayed development was most likely due to the exposure and rearing of embryos in constant dark conditions, as well as lowered temperature, which were used to prevent degradation of the photolabile, and temperature sensitive, carotenoids (see 2.2. *Zebrafish embryo teratogenicity assay*). Similar delays in development related to reduced light and temperature have been previously reported by several authors (Schirone and Gross, 1968; Billotta, 2000; Villamazar et al., 2014; Di Rosa et al., 2015). Notably, for example, Billotta (2000) investigated effects of light on various aspects of zebrafish embryo development, and found that embryos raised in constant dark were generally “developmentally delayed,” and specifically “were still not hatched by 7 dpf.” Subsequent studies have, likewise, shown developmental delay due to rearing in constant dark including various consequent post-larval effects (Villamazar et al., 2014; Di Rosa et al., 2015). Alongside delayed development, relatively high embryotoxicity within 1–2 dpf was observed for all embryos (including controls) reared in constant dark, as specifically evidenced by coagulation, and independent of the generally lethal deformities. Embryo survival within 1–2 dpf was approximately 61% ± 8% for controls and did not significantly differ for embryos exposed to carotenoids (or

retinoids). However, despite the generally delayed development, and relatively high mortality, comparison to untreated controls enabled teratogenicity to be clearly distinguished both quantitatively and qualitatively.

Qualitatively, the developmental toxicity varied between carotenoids, and with exposure concentration (Fig. 5). By 3 dpf, for example, embryos exposed to both **1** and **2** at 5 μM and greater showed pronounced developmental dysfunction (Fig. 5C–F) compared to controls (Fig. 5H–G). Developmental deformities observed were sufficiently severe to be distinguished as lethal by 3–5 dpf. Lutein (Fig. 5I) and canthaxanthin (Fig. 5K) which reached levels of teratogenicity equivalent to **1** and **2** by 3 dpf (i.e., 100% deformity at 15 μM ; Fig. 6), likewise, shared several observable features of severe developmental dysfunction with the xanthophyll glycosides. These features included impaired development along the anterior-posterior axis, and specifically consequent head and eye hypoplasia and truncation of the tail, as well as pericardial edemas at 5 dpf (Fig. 5). Inhibition of the anterior-posterior development by carotenoids is notably reminiscent of, albeit less severe than, the effects of RA exposure (Fig. 5A–B) which showed severely arrested development along the body axis. In contrast, embryos exposed to zeaxanthin at the same developmental stage (e.g., 3–5 dpf) - and higher exposure concentrations (e.g., 60 μM) - developed normally, and were indistinguishable from controls (Fig. 5J) as reflected, likewise, in the quantitative analyses. Teratogenicity of β -carotene which only manifested at ~5 dpf (Fig. 6) seemingly differed from **1** and **2**, and other xanthophylls (i.e., lutein, canthaxanthin). Although impairment of head/tail development was observed, it was perhaps less pronounced, and instead, a range of pericardial - and other - edemas were observed (at 5 dpf; Fig. 5L).

Quantitatively, toxicity among carotenoid tested also varied significantly (Fig. 6). Statistically significant development toxicity (i.e., percent deformity) was observed within 2–3 dpf for **1** and **2** at the three highest concentrations (15, 30 and 60 μM) tested. Percent deformity was additionally significant by 3 dpf for embryos exposed to the keto-variant (**2**) at the next highest concentration (5 μM). At 2 dpf, no other carotenoids were toxic with the notable exception of the diketo-xanthophyll, canthaxanthin, for which significant development toxicity was observed at the highest test concentration (approximately 89% for 60 μM ; Fig. 6). By 3 dpf, however, teratogenicity of both canthaxanthin and the dihydroxylated xanthophyll, lutein, reached levels comparable to both **1** and **2** (i.e. 100%) at all three of the highest concentrations tested (Fig. 6). Developmental toxicity was sufficiently severe, in all cases, such that deformities were effectively lethal, and EC_{50} (for teratogenicity) and LC_{50} were, therefore, equivalently calculated ($\pm$ standard error) at 5 dpf for myxol glycosides **1** and **2** as $2.4 \pm 0.9 \mu\text{M}$ and $1.8 \pm 0.2 \mu\text{M}$, respectively; and for xanthophylls, lutein and canthaxanthin, respectively, as $4.7 \pm 3.0 \mu\text{M}$ and $8.7 \pm 1.8 \mu\text{M}$. Interestingly, no teratogenicity was observed for the chemically related xanthophyll, zeaxanthin, during the 5-day exposures studies (Fig. 6). Developmental toxicity was observed for β -carotene, but only at the highest concentrations tested (30 and 60 μM), and only after 5 dpf ($\text{EC}_{50}/\text{LC}_{50} = 23.5 \pm 8.9 \mu\text{M}$). Similarly, no teratogenicity was observed for the apocarotenoid, β -apo-8'-carotenal, at 3 dpf; slight developmental toxicity (~22%) was observed at 5 dpf, but this was not significantly different from controls. The relatively

limited teratogenicity of β -carotene is particularly notable as it is recognized as the primary substrate for symmetric cleavage by BCO1, subsequently leading to production of RA (Goodman et al., 1967; Lindqvist and Andersson, 2002).

4. Discussion

4.1. Isolation and identification of myxol glycosides as teratogenic metabolites

Bioassay-guided fractionation enabled purification of three chemically related xanthophylls from *C. raciborskii* as teratogens in the zebrafish embryo model, and subsequent chemical characterization identified the bioactive metabolites as myxol glycoside (**1**), along with its 4-keto (**2**) and 4-hydroxy (**3**) congeners. Frequently referred to as “myxoxanthophyll” in the literature (Hertzberg and Liaaen-Jensen, 1969), myxol glycoside (**1**) has been previously identified from cyanobacteria, including *C. raciborskii* (Varkonyi et al., 2002), and specifically shown to be required for integrity of thylakoid membranes (Mohamed et al., 2005). In contrast, 4-ketomyxol glycoside (**2**) has not been previously identified from *C. raciborskii*, but has been identified (Schlüter et al., 2004; Takaichi et al., 2005) in other recognized toxigenic genera of cyanobacteria (e.g. *Anabaena*, *Nostoc*, *Nodularia*), and even been proposed (Schlüter et al., 2004) as a proxy of the presence of these toxin-producing species (e.g. *Nodularia spumigena*). Similarly, the 4-hydroxy variant has, likewise, been found among various cyanobacterial genera/species including quite recently *C. raciborskii* (Mehnert et al., 2012). Despite widespread – and, in fact, exclusive – occurrence of the xanthophyll glycosides among cyanobacteria, the teratogenicity observed in the current study is the first report of any bioactivity, specifically relevant to potential toxicity, of these metabolite.

4.2. Proposed pro-retinoid mechanism for teratogenicity of myxol glycosides

Although this is the first report of teratogenicity associated with cyanobacterial (or any) carotenoids, notable members of the group are well-recognized as “pro-retinoids” serving as substrates for a biochemical diversity of so-called *carotenoid cleavage enzymes* (CCE) which convert carotenoids to various apocarotenoids and retinoids. As the primary example, β -carotene is converted to retinaldehyde via oxidative cleavage (Fig. 7) by a family of oxygenases, and specifically the so-called β -carotene 15,15'-oxygenases (BCO), which are widely distributed among vertebrates and invertebrates (Von Lintig and Vogt, 2000; Shete and Quadro, 2013). Although β -carotene is primarily recognized as the substrate for BCOs, studies have demonstrated the oxidative cleavage of various other carotenoids by these enzymes. In particular, relatively high substrate specificity for β' carotene has been shown for members of the BCO1 sub-type, whereas the less well characterized BCO2 sub-type is apparently capable of the oxidative cleavage of diverse carotenoids including carotenes and xanthophylls (Kiefer et al., 2001; Mein et al., 2011; Hu et al., 2006; Amengual et al., 2011; Sui et al., 2013; De la Seña, 2014). Specifically, BCO2 cleaves carotenoids “eccentrically” as a 9',10'-oxygenase (as opposed to the symmetrical, or “centric,” cleavage by BCO1), giving rise to an apocarotenoid, β -apo-10'-carotenal (Fig. 7). The apocarotenoid can be subsequently converted to retinoids by one of two proposed mechanisms. It has been shown that apocarotenoid acids (via interconversion from apocarotenal) can undergo β -oxidation to retinoic acid (Wang et al., 1996). Alternatively, it has been more recently shown that BCO1

can, in fact, cleave a diversity of apocarotenals (including β -apo-10'-carotenal) to retinal with an efficiency similar to that of β -carotene (Amengual et al., 2013). Retinaldehyde can, in turn, be converted to other retinoids including retinoic acid (RA) and retinol (i.e., Vitamin A) via retinaldehyde dehydrogenases (Raldh) and retinol dehydrogenase (RoDH) steps, respectively (Fig. 7).

Retinoic acid - including all *trans* (ATRA) and 9-*cis* (9-*cis*RA) isomers - are essential signaling molecules in embryonic development. Binding of ATRA and 9-*cis*RA to so-called retinoic acid receptors (RAR) and "retinoid X" receptors (RXR), respectively, enables formation of heterodimers (RAR/RXR) which, in turn, mediate expression of a suite of genes via binding to *retinoic acid response elements* (RARE) within the genome (Fig. 7). Perhaps most notable among these genes are the highly conserved homeotic (*Hox*) genes that are fundamental to anterior-posterior patterning in development of embryos. Despite its role as key signaling molecule in embryonic development, RA and several of its variants are, therefore, also well-recognized teratogens (Hermann, 1995; Collins and Mao, 1999; Selderslaghs et al., 2009; Wang et al., 2014), and production of RA in developing embryos is, thus, tightly regulated. Regulation at this level specifically occurs via oxidative degradation by a cytochrome P450 (i.e., cyp26) that produces more polar metabolites (e.g. 4-hydroxy-RA, 4-oxo-RA).

In the present study, we evaluated a representative series of retinoids including ATRA, retinol and retinal. Teratogenicity of RA is directly linked (Minucci et al., 1997) to interaction with RAR (and, following dimerization with RXR, the RARE promoter). In contrast, it has been shown (Repa et al., 1993) that retinal shows essentially no binding affinity to RAR, whereas retinol does compete with RA for binding to RAR, but with a much lower (i.e., approximately 4- to 7-fold) affinity. The differential activity of the retinoids is generally consistent with the well-described interconversion of the retinoid congeners (Fig. 7; see Von Lintig et al., 2005). Specifically, retinal can be directly transformed by Raldh to RA, whereas retinol is reversibly converted, specifically by RoDH, to retinal that can, in turn, be enzymatically transformed (by Raldh) to RA. We propose, therefore, that this tripartite interconversion to form teratogenic RA (i.e. retinol $\leftrightarrow$ retinal $\rightarrow$ RA), along perhaps with possible direct binding of retinol, explains the three-tiered teratogenicity observed in the zebrafish embryo.

A number of very recent studies have pointed to the apparent production of retinoids by cyanobacteria and other freshwater algae (e.g., "green algae," or Chlorophyta), and have suggested a role of the teratogenicity of these cyanobacterially derived retinoids in the toxicity of blooms including impacts on aquatic vertebrate populations (Wu et al., 2012, 2013; Jonas et al., 2014, 2015; Javurek et al., 2015). In a particularly notable study, Wu et al. (2012, 2013) identified an array of retinoids from algal blooms in the notoriously eutrophic Lake Taihu (China), as well as thirty-nine algal and cyanobacterial cultures isolated from the lake and diverse range of other freshwater sources. Evaluation of cyanobacterial and algal (including chlorophyte, diatom and euglenophyte) strains in this study showed that most (>82%) strains were capable of producing retinoids including retinal, and various isomers (e.g., 13-*cis*, 9-*cis* and all *trans*) of RA and 4-oxo-RA. Subsequent studies have, likewise, identified "retinoid-like" bioactivity of various cyanobacteria including observation of

teratogenicity in the zebrafish embryo model (Jonas et al., 2014, 2015). In one study, it was observed that retinoid activity could not be explained by the presence of the detected retinoids, and suggested that other metabolites contribute to this bioactivity (Javurek et al., 2015).

It is perhaps not surprising that cyanobacteria are capable of producing retinoids: prior studies have, in fact, identified and characterized a family of apocarotenoid oxygenases (ACOs) from cyanobacteria, and have shown the ability of these enzymes to cleave diverse apocarotenoids including the consequent production of retinal (Scherzinger et al., 2006; Scherzinger and Al-Babili, 2008).

Indeed, it has been suggested, but not shown, in these more recent studies that carotenoids might be the biosynthetic source of retinoids produced cyanobacteria (Wu et al., 2013).

The observed teratogenicity of **1–3** specifically led to the hypothesis that myxol glycosides act as pro-retinoids, and specifically provide a substrate for BCO cleavage, and subsequent production of retinoids. Indeed, the observed teratogenicity in zebrafish embryos exposed to **1–3** bears striking quantitative and qualitative resemblance to the teratogenicity previously observed (Hermann, 1995; Jonas et al., 2014) – and repeated in the current study - for RA in this model system. Although more severe in the case of RA exposure, embryos exposed to **1**, **2** and RA were characterized by clearly impaired development of the body axis, and consequent hypoplasia of the head and eye, as well as truncation of the tail (see Fig. 5). And, in fact, this feature is a hallmark of RA teratogenicity specifically owing to its role in anterior-posterior patterning via *Hox* gene regulation (Waxman and Yelon, 2009). Whereas retinal, followed by enzymatic conversion to RA, is the established product of the oxidative cleavage of β -carotene, oxidative cleavage of xanthophylls is expected to yield hydroxy- and keto-variants of these retinoids. Hypothesized products of **1** and **2** would thereby include, 3-hydroxy-RA and 3-hydroxy-4-oxo-RA, respectively. Notably, 4-hydroxy and 4-oxo-RA, as products of the oxidation of RA by cyp26, were previously shown to act as potentially teratogenic retinoids in the zebrafish and other models (e.g., *Xenopus* embryos), and specifically act via interaction with RAR, and subsequent expression of *Hox* genes (Pijnappel et al., 1993; Hermann, 1995). The hypothesized products from oxidative cleavage of **1** and **2**, however, remain to be confirmed in future studies.

In further support of pro-retinoid hypothesis, and toward a model of teratogenicity, a series of carotenoids and apocarotenoids were evaluated in the zebrafish embryo model. Notably, all of the carotenoids (except **1** and **2**) have been previously detected - along with retinal, and relatively small quantities of retinol – as endogenous components in the yolk of zebrafish eggs (Lampert et al., 2003). Moreover, all of the carotenoids (with the exception of lutein, but including **1** and **2**) have been identified from cyanobacteria. In the present study, variable teratogenicity of several of the carotenoids was observed. Moreover, the developmental toxicity showed considerable quantitative and qualitative variation with respect to variant, effective concentration and embryonic stage at which teratogenicity was observed (Figs. 5 and 6).

Further studies are clearly needed, however, we propose based on these observations that the differential teratogenicity of the carotenoids during development is related, in part, to differential expression of BCO subtypes during embryo development. Currently two BCO subtypes, i.e. BCO1 and BCO2, are recognized, and both have been isolated from zebrafish embryos (Lampert et al., 2003; Lobo et al., 2012a). The latter is compartmentalized within mitochondria, whereas the former is cytoplasmic (Amengual et al., 2011; Lobo et al., 2012a; 2012b; Palczewski et al., 2014). The two subtypes have different substrate specificities with BCO1 showing a high specificity for β -carotene, and a more general requirement of a β -ionone ring (Lindqvist and Andersson, 2002), whereas BCO2 is able to cleave a relatively wide range of carotenoids notably including both hydroxylated and 4-oxo-substituted xanthophylls (i.e. lutein, zeaxanthin, canthaxanthin). In fact, several lines of evidence suggest that BCO2 preferentially cleaves carotenoids containing 3-hydroxy-ionone ring, and β -carotene has been shown to be a generally poor substrate for BCO2 (Mein et al., 2011; De la Seña, 2014 and De la Seña et al., 2016). To date, no study has directly evaluated either BCO subtype with respect to the xanthophyll glycosides, although cleavage of myxoxanthophyll by both other CCE (e.g., ACO) from cyanobacteria has been demonstrated (Scherzinger et al., 2006; Scherzinger and Al-Babili, 2008).

Alongside substrate specificity, we propose that stage dependent occurrence of BCO subtypes may underlie the observed temporal patterns of the teratogenicity of the carotenoids. The established mitochondrial localization of BCO2 (Lobo et al., 2012a; 2012b) might, in particular, imply that this subtype would be present during early stages of embryo development. Mature oocytes of the zebrafish contain remarkably high densities of maternally derived mitochondria. In a very recent study (Otten et al., 2016) the number of mitochondrial DNA (mtDNA) copies, for example, was found to be on the order of 20×10^6 copies per cell compared to five orders of magnitude fewer, i.e., 100–1000 copies, in somatic cells. In the proposed model, therefore, teratogenicity of the xanthophylls would result from sequential cleavage: during early stages of development, mitochondrial BCO2 would cleave xanthophylls to corresponding apocarotenoids, and subsequent CCE (i.e., ACO or BCO) activity, along with possible β -oxidation, would convert the resulting apocarotenoids to teratogenic retinoids. This general mechanism has, in fact, been demonstrated recently in a mouse model (Amengual et al., 2013). On the other hand, β -carotene is a relatively poor substrate for BCO2 (De la Seña, 2014), and this may explain, therefore, very low teratogenicity of this carotenoid until later stages of development (i.e., 5 dpf) at which point BCO1 becomes more widely expressed in the zebrafish embryo (Lampert et al., 2003). Although BCO1 is expressed starting at 14 somite stage (Lampert et al., 2003), expression is localized to the yolk/embryo interface, and widespread expression (e.g. gut, liver) is only observed at ~3–4 dpf (Lampert et al., 2003). Also consistent with this model is the observation of very slight teratogenicity at 5 dpf for β -apo-8'-carotenal which has been shown to be cleaved by BCO1, but with a much lower efficiency than β -carotene (De la Seña et al., 2013).

The most notable discrepancy in this model is the teratogenicity of lutein, but apparent lack of teratogenicity of its stereoisomer, zeaxanthin. It has been previously demonstrated in mammalian and avian systems that BCO2 is capable of eccentric cleavage of both zeaxanthin and lutein, to corresponding 3-hydroxy-apo-10'-carotenals which, in turn, are

substrates for subsequent enzymatic or non-enzymatic (i.e., β -oxidation) conversion to retinoids (Amengual et al., 2011; Mein et al., 2011; De la Seña, 2014). That said, studies generally suggest significant phylogenetic diversification of BCO subtypes which likely relates to differential utilization of carotenoids in different systems (e.g., fish, avian, mammalian). In particular, recent studies have specifically suggested an evolutionary divergence of BCO2 isoforms among teleost fish (Helgeland et al., 2014). As opposed to mammalian systems with two identified subtypes, studies in a teleost fish model (i.e., Atlantic salmon) – and subsequent evaluation of sequence databases - identified at least five isoforms, including 2 BCO1 and 3 BCO2, resulting from genome duplication events, and presumptive subsequent sub-functionalization, in the teleost evolutionary lineage (Helgeland et al., 2014). Interestingly, these authors specifically proposed a functional divergence of BCO subtypes among teleost fish related to the high diversity of carotenoids, particularly from algae and cyanobacteria, present in aquatic systems (Helgeland et al., 2014). Further characterization of CCE in the zebrafish model might, therefore, clarify the discrepancy between teratogenicity of lutein and zeaxanthin observed here. In this regard, it is particularly noteworthy that zeaxanthin, but not lutein, is produced by cyanobacteria and other algae.

Finally, in addition to the proposed role of differential substrate specificity of BCO subtypes, it is also likely that solubility of carotenoids contributes to the variable teratogenicity observed in the present study. Carotenoids are generally lipophilic compounds. Of the carotenoids, the carotenes, such as β -carotene, are the most lipophilic (and thus least water soluble), whereas oxygen-containing xanthophylls (owing to the presence of hydroxyl and keto functional groups) are more polar and, although still lipophilic, relatively more soluble in the aqueous phase. Extending this trend to the xanthophyll glycosides, the presence of the sugar moiety would clearly even further increase hydrophilicity, and therefore, the potential availability of these compound (i.e. **1–3**) in the aqueous assay medium. Indeed, certain carotenoid glycosides, such as crocin, are the few known water-soluble variants (Háda et al., 2012). Consistent with this, **1–3** were, in fact, isolated in the most polar fractions from normal-phase chromatography (see 2.3. *Extraction and purification of carotenoids by bioassay-guided fractionation*). Increased water-solubility (and, thus, dissolved concentration) of the xanthophyll glycosides may, thereby, contribute to the particularly rapid and potent toxicity of **1** and **2**. With respect to the ecotoxicological implications, the increased water-solubility of the xanthophyll glycosides might similarly support a relatively larger contribution of these carotenoids in aquatic systems, and specifically as a component of the consequently *dissolved fraction* in these systems.

4.3. Environmental relevance of the teratogenicity of carotenoids

Identification of the cyanobacterial xanthophyll glycosides (**1–3**) as teratogenic in the zebrafish model, and the teratogenicity of carotenoids more generally, suggest a potential impact of these compounds on aquatic animal populations including reduced recruitment and survival. However, relevance of the observed teratogenicity of carotenoids to possible effects on natural populations remains to be clarified with respect, in particular, to environmental concentrations.

Carotenoids associated with *particulate* (i.e., algal/cyanobacterial cell) fractions of aquatic systems are widely utilized to quantify both algal/cyanobacterial density generally, and as so-called “pigment markers,” specific taxa (e.g., cyanobacteria, chlorophytes, diatoms, etc.). Unfortunately, however, very little is known with respect to the concentration of carotenoids in the *dissolved* fraction of aquatic systems (as a direct route of exposure). Although carotenoids are generally considered lipophilic, it is hypothesized (as discussed above) that xanthophyll glycosides will have significantly higher water-solubility, and thus concentrations in dissolved fractions.

In the absence of specific data (i.e., measurements) regarding dissolved concentrations, potential contribution of carotenoids from cyanobacteria in aquatic systems can be generally estimated. Typical concentration of carotenoids, specifically including the myxol glycosides, have been conservatively estimated in the range of 0.1–0.5% dry weight (Montero et al., 2005; Schagerl and Müller, 2006). Density of cyanobacterial blooms are frequently cited, specifically using chlorophyll A (Chl A) as a proxy, to be as high as 3000 µg Chl A L⁻¹ (Zohary and Roberts, 1990); and, using an accepted conversion factor of 0.5–1% Chl A per dry weight of biomass, can be translated to approximately 0.3 g cyanobacterial biomass per liter. Contribution of carotenoids from cyanobacteria can, therefore, be calculated in the range of approximately 0.4–3 µM (using a molar mass range of 537–747 g mol⁻¹ for β-carotene and myxol glycosides, respectively). Although such carotenoid concentrations might be limited to relatively dense bloom conditions, the calculated range would include concentrations approaching the lower levels (i.e., 1 and 5 µM) tested in the current study. While the specific contribution to carotenoids in the dissolved fraction remains to be seen, it is generally assumed to be affected by both degradation (or other forms of removal), and conversely, possible accumulation within the dissolved fraction. Realistic concentrations may, therefore, be lower (due to degradation/removal) or higher (due to accumulation).

Finally, it should be noted that exposure concentrations in the present study represent *relatively high* levels of toxicity. Concentrations in the study were associated with toxicity specifically on the order of the EC₅₀/LC₅₀ (i.e., 50% severe deformity/lethality) or greater. These concentrations would not be necessary for carotenoids to contribute to reduced populations. Otherwise stated, concentrations equivalent to, for instance, the EC₁₀/LC₁₀ (which would reduce population viabilities by 10%) would be sufficient to have profound effects on the natural populations. Clearly further study is needed to understand the relevance of the carotenoids, particularly in relation to effective environmental concentrations.

5. Conclusions

The present study shows that xanthophyll glycosides, including myxol-2'-glycoside and its 4-keto and 3,4-dihydroxylated congeners, widely produced by cyanobacteria, can act as potent teratogens in the zebrafish embryo model. It is specifically hypothesized, based on these findings, that xanthophyll glycosides act as proretinoids similar to other carotenoids which are well studied in this regard. The comparative toxicology studies suggest that teratogenicity can be largely, but with exceptions (i.e., zeaxanthin), be potentially explained by the current knowledge of substrate specificity, and perhaps developmental patterns of the

localization and expression, of the recognized sub-types of CCE associated with oxidative cleavage of carotenoids to retinoids in the zebrafish model.

The teratogenicity of carotenoids observed in the present study suggests a potentially direct link between cyanobacterial abundance and toxic potential in freshwater systems including, in particular, effects on aquatic vertebrate populations (i.e., fish, amphibians). Unlike other recognized “cyanotoxins,” carotenoids are not phylogenetically restricted, and produced by all cyanobacteria which are obligate producers of carotenoids both as accessory pigments, and for protection against associated photooxidative stress. And, indeed, these algae are the primary autochthonous source of carotenoids in freshwater systems. Although cyanobacteria produce several carotenoids, the xanthophyll glycosides are generally recognized as unique to (and widely distributed within) the phylum (Schlüter et al., 2004). It has been suggested that carotenoids may be biosynthetic precursors of retinoids recently identified from cyanobacteria (Wu et al., 2012; Scherzinger et al., 2006). As such, carotenoids arguably represent a two-tiered source of teratogenic metabolites including both endogenous biosynthesis of retinoids by cyanobacteria, and *in situ* conversion of carotenoids to retinoids by vertebrate enzymes (i.e. BCOs, and other CCEs).

The toxicity of the carotenoids toward embryonic stages of the zebrafish, as an environmentally relevant (i.e., freshwater teleost) aquatic vertebrate model specifically underscores the potential impact of this toxicity to aquatic vertebrate populations. The presence of similarly teratogenic retinoids in cyanobacterial blooms has, in fact, been recently proposed as a factor in the local declines, as well as increased frequencies of developmental deformities, among aquatic vertebrate populations (Wu et al., 2012, 2013). It is, likewise, possible that carotenoids may similarly contribute to such effects on freshwater ecosystems either directly and/or indirectly (as biosynthetic precursors to retinoids). Perhaps most notable, in this regard, is concurrence between unprecedented declines in amphibian populations worldwide, and temporally coincident (i.e., decadal) global increases in the proliferation of cyanobacteria in freshwater and coastal systems. As to the former, a preponderance of evidence points to global decline in amphibian populations since the 1950s, and although several hypothesis have been put forth to explain this rapid disappearance, there remains no clear explanation (Houlahan et al., 2000; Blaustein and Kiesecker, 2002; Stuart et al., 2004). On the other hand, an emerging body of evidence suggests increased frequency and persistence of blue-green algae in freshwater and coastal systems within an equivalent time-frame. This increased cyanobacterial proliferation in aquatic is perhaps driven, as it has been argued (e.g., Paerl and Paul, 2012), by accelerated eutrophication (i.e. nutrient inputs), global climate change (i.e. warming) and various related factors. Although future study is clearly needed, the potential contribution of teratogenic carotenoids as a factor in these well documented declines in aquatic vertebrates is a compelling notion.

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HIGHLIGHTS

- Myxol glycosides from cyanobacteria were identified as teratogenic in the zebrafish embryo model.
- Comparison to other carotenoids and retinoids shows congener-specific teratogenicity.
- A model of teratogenicity of carotenoids as pro-retinoids is proposed.
- Relevance of teratogenicity to declines in aquatic vertebrates is discussed.

Fig. 1.
Proposed structure of carotenoids (**1–3**) isolated in the present study.

Fig. 2.

Carotenoids and retinoids evaluated for teratogenicity in the zebrafish embryo model.

Carotenoids include carotenes (β -carotene), and xanthophylls (zeaxanthin, lutein, canthaxanthin), as well as an apocarotenoid (apo-8'-carotenal). Retinoids include the three common products of oxidative cleavage of β -carotene (retinal, retinoic acid [RA] and retinol).

Fig. 3. Bioactive component (**3**) identified from *C. raciborskii* 121-1. Detection by HPLC, coupled to PDA detection, identified a single bioactive component with a UV/Vis spectrum indicative of aphanizophyll.

Fig. 4.

Comparison of the teratogenicity of retinoids in the zebrafish embryo model. Shown are percent deformities ($n = 3$, 5 embryos per replicate) for concentrations ranging from 1 to 60 μM of RA (A), retinol (B) and retinal (C) at 3 dpf. Error bars represent one standard error above or below means. Statistically significant differences, compared to untreated controls, by Fisher Exact Test: ** $p < 0.005$.

Fig. 5.

Teratogenicity of carotenoids and retinoic acid. Shown are examples of the teratogenicity of 1 μ M RA at 3 dpf (A) and 5 dpf (B) with embryos exhibiting severely arrested development of the body axis including head and eye hypoplasia and truncated tail; 5 μ M **1** at 3 and 5 dpf (C and D, respectively) with head and eye hypoplasia, truncated tail and pericardial edema; and **2** (E and F) at 3 and 5 dpf, respectively, with head and eye hypoplasia and truncated tail compared untreated controls (G and H) at the same time points. For comparison between carotenoid variants, the teratogenicity is shown for embryos exposed to: 30 μ M lutein (I) with head and eye hypoplasia and truncated tail; 60 μ M zeaxanthin (J) with little effect at 3 dpf; 30 μ M canthaxanthin at 3 dpf (K) with head and eye hypoplasia and truncated tail; and 30 μ M β -carotene at 5 dpf with truncated tail and pericardial edema (L). Abbreviations: hh = head and eye hypoplasia, tt = truncated tail and pe = pericardial edema.

Fig. 6.

Comparison of the teratogenicity of carotenoids in the zebrafish embryo model. Shown are percent deformities ($n = 3, 5$ embryos per replicate) for concentrations ranging from 1 to 60 μM of **1** (A), **2** (B), β -carotene (C), lutein (D), zeaxanthin (E), canthaxanthin (F) and β -apo-8'-carotenal (G) at 2, 3 and 5 days post-fertilization (dpf). Structures of carotenoids are shown in Fig. 2. Error bars represent one standard error above or below means. Statistically significant differences, compared to untreated controls, by Fisher Exact Test: * $p < 0.05$, ** $p < 0.005$.

Fig. 7.

Oxidative cleavage of carotenoids to retinoids, and subsequent role in gene expression. Shown is the well-described conversion of β -carotene to retinal, retinol and retinoic acid, and the proposed conversion of the cyanobacterial xanthophyll glycosides (**1–3**) isolated in the present study to hydroxyl- and oxo-retinoids. Oxidative cleavage occurs either via centric cleavage to produce retinals by BCO1, or eccentric cleavage to apocarotenoids (e.g. apo-8'-carotenal) by BCO2. Apocarotenoids can be converted to retinoids by either β -oxidation, or cleavage by BCO1, as described. Retinal is converted enzymatically to either retinoic acid (RA) by retinaldehyde dehydrogenase (Raldh), or reversibly to retinol by retinol dehydrogenase (RoDH). Binding of all-trans and 9-cis RA isomers (ATRA and 9-cisRA) to the retinoic acid and "retinoid X" receptors (RAR and RXR, respectively) enables formation of a heterodimer (i.e. RAR/RXR). The dimer, in turn, binds to retinoic acid response element (RARE) sequences in various gene (e.g. *Hox*) promoters. Concentrations of RA in developing cells are controlled via oxidation (and elimination) by a cytochrome P450, and specifically cyp26, to more polar 4-hydroxy and 4-oxo retinoids.