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Title: Toxicity of Fungal Pigments from *Chlorociboria* spp. and *Scytalidium* spp.

Abstract Approved:

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Commercial methodologies for producing fungal pigments are of worldwide interest due to the desire to move away from synthetic dyes. *Chlorociboria* species and *Scytalidium* species have been reported to produce sufficient yields of pigments for commercial production and have attracted special attention because of their use in spalted wood applications. However, there are few data about the toxicity of these pigments on humans or the ecosystem. The main objective of this thesis was to examine fungal pigment mixture toxicity and its effects on living organisms using a zebrafish embryo acute toxicity bioassay. Pigment mixtures from wood agar cultures and liquid malt media were screened. There were significant adverse effects from both the DCM-extracted pigment and the liquid malt medium although there was variability in the toxicity endpoints. The results from this study suggest that all dichloromethane (DCM) pigment extracts followed a dose/ response curve and caused higher mortality in higher concentrations after a short time of exposure except the DCM-red pigment extract which follow a non monotonic dose/response curve. The response from both DCM pigment extracts and liquid malt pigment depended on the solubility and bioavailability factors in the water. Overall, the results indicate that the pigments extracted from these fungi are likely toxic to humans. However, as no completely purified compounds were tested, it is possible that other secondary fungal metabolites and wood extractives that were also retrieved during the extraction process might also have played a role in the toxicity.

Toxicity of Fungal Pigments from *Chlorociboria* spp. and *Scytalidium* spp.

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Badria H. Almurshidi, Author

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1. Introduction and Literature Review

Colors, both natural and synthetic, permeate our lives. Synthetic pigments commonly are found in food, cosmetics, medicine, clothes, and other products (Hamlyn, 1998). One of the most abundant natural colors is chlorophyll, used in photosynthesis, which is important for converting light to chemical energy. Another common class of natural pigment is the quinone, which is derived from plants. Melanin, another pigment, provides protection from ultraviolet degradation in humans, plants, and fungi. Most importantly, melanin has been noted to protect fungi from toxins (Wheeler & Bell, 1988).

Fungi are eukaryotic microorganisms that are known to inhabit almost all ecological niches on earth (David, 1986). Fungi produce secondary metabolites that are used for a variety of biological activities other than normal growth and development. They include compounds that inhibit growth or development of other organisms in the surrounding environment. Many fungal secondary metabolites are therefore of great interest for agro chemistry, pharmaceuticals, and the food industry (Wheeler, 1983). Much research has been conducted to investigate the commercial value of some of the secondary metabolites of fungi. Specifically, there is interest in using natural fungal pigments in the textile industry as a replacement for synthetic dyes (Kumar, 2009; Harman, 2013).

1.1 Description of Fungi

More than 70,000 fungal species have been described in the world, although it has been suggested that there could be up to 1.5 million species (Blackwell, 2011). The most common types of fungi include rusts, mushrooms, smuts, truffles, molds, morels, and yeasts (Zabel & Morrell, 1992). Fungi are involved in the nutrient cycles on earth via the breakdown of dead organic matter. The symbiotic relationship between plants and fungi, such as mycorrhizae, is very important to plants. The fungi in this relationship serve to supply the plants with essential nutrients for their growth. Fungi can

have detrimental effects, causing ringworm, athlete's foot, and several other diseases in human beings (Brown, 2012-a; Brown, 2012-b; Olasode, 2014). Fungi can also cause plant diseases such as rust, leaf rot, and stem rot (Fisher, 2012)

Fungi require specific conditions to flourish. If these conditions are hindered, then their growth is affected. The moisture content of wood is critical for wood-inhabiting fungi. For fungi to thrive, the moisture content of wood fibers should be above 30%, which is just above the fiber saturation point. At this moisture level, the lumen is not filled with water, but the cell walls are saturated. Although fungi can grow in wood with a moisture content of 20% (Hoadley, 2000), fungal growth diminishes below this level.

Temperature is also a crucial condition for fungal growth, with the temperature range for development being 0–45°C; although the ideal temperature varies among species (Zabel and Morrell, 1992). Outside this temperature range, fungal growth is affected. Nevertheless, some fungi have developed forms of temperature resistance and can develop even under extreme temperatures that are well outside their optimum. Temperature variations mainly influence fungal enzymes, which function optimally within an ideal temperature range. Periods of extreme temperature exposure can affect fungal survival (Chang, 1999).

Oxygen enhances fungal development, and without it, fungi in wood cannot grow and develop. Waterlogged wood has little or no oxygen; hence, fungal development is hindered. High moisture content reduces fungal development by decreasing the amount of oxygen in the wood (Cartwright and Findlay, 1958; Boddy, 1983).

1.2 Fungal Pigments

Fungal pigments are chemical compounds that play an important role in the absorption of visible light in a wavelength range between 400–700 nm. These pigments are synthesized as secondary metabolites. Pigments from filamentous fungi include melanins, carotenoids, flavins,

quinones, phenazines, violacein, and monascins (Lichter & Mills, 1998). Food grade pigments from filamentous fungi are available commercially but not commonly utilized. They include *Monascus* pigments, a pink red from *Penicilium oxalicum*, riboflavin from *Ashya gossypii*, and lycopene and carotene from *Backeslea trispora*. The yellow and red polyketide pigments harvested from *Monascus* spp. are used as food colorants in the form of pigment extracts (Jůzlová, 1996).

Fungal pigments are classified into four different classes via the chemical structure of the chromophore. Some have chromophores with conjugated systems such as anthocyanins, carotenoids, betalains, and caramel. Other fungal pigments are categorized according to the structural characteristics of their natural pigments such as the isoprenoid derivatives: carotenoids and iridoids. However, *N*-heterocyclic compounds are different from tetrapyrroles and are made up of the purines, pterins, flavins, phenazines, phenoxazines, and betalains. There are also benzopyran derivatives (oxygenated heterocyclic compounds) called anthocyanins (Mapari, 2009). Of specific interest for the current study are the quinones such as benzoquinone, naphthoquinone, and anthraquinone. Melanins are also frequently produced by fungi.

A number of species in the Ascomycota are well known for their pigments production. Of particular interest are the genera *Chlorociboria* and *Scytalidium*, in which the pigments have significant potential for the dye industry (Weber, 2014). *Chlorociboria aeruginascens* and *Chlorociboria aeruginosa* are globally distributed and contain a quinone pigment that is known as xylindein (Futoshi et al. 1996, Shibata et al. 2007). Wood colored by these fungi is commonly used in decorative woodwork such as Tunbridgeware and marquetry (Hartig, 1878; Berkely, 1860). The pigment extracted from *Scytalidium cuboideum* is also used in wood spalting, where spalting is defined as any color formed *inside* wood produced by a fungus or fungi (Chidester, 1940; Robinson et al. 2007a).

1.3 Natural and Fungal Color in the Textile Industry

Synthetic dyes are thought to have a number of advantages over natural dyes, such as color fastness, reproducibility, ease of use, and availability. However, there is a growing interest in the use of natural dyes in the textile industry for coloration. Fungi are known to produce a number of stable colorants (Sharma, 2010). Some of the most commonly utilized are the anthraquinone compounds. These compounds include melanin, delphinidin, and volatile organic compounds (Gill, 1987; Gulrajani, 1992; Cappuccino, 2004; Dufosse, 2006; Sharma, 2010).

The British Textile Technology group investigated the production of fungal pigments as well as their use as textile colorants (International Congress on Photobiology, 1974). They stated that three conditions must be met for fungal pigments to effectively replace synthetic pigments:

1) The fungal growth medium should not contain expensive chemicals or those that are toxic to humans or other organisms.

2) The fungal culturing and extraction processes should be conducted under low temperatures (around 20°C) in contrast to the high temperatures used for synthetic dyeing.

3) The manufacturing process for fungal pigments must occur at a neutral pH, which is in contrast to the highly acidic or alkaline conditions used for synthetic dyeing.

Fungal color is widely used in the textile industry for dyeing yarns that are then woven into clothes or other usable forms (Sharma, 2010). Studies have shown that cotton clothes have a high absorption rate for natural dyes (Amaranthus, 1998).

1.4 Spalting

The history of spalting can be traced back to the 15th century, where spalted wood was used for intarsia woodworks. Its use has continued through the centuries, in marquetry, parquetry,

Tunbridgeware, woodturning, and furniture (Windisch-Graetz, 1983; Wilmering, 1999; Blanchette et al., 1992; Michaelsen, 1992).

In the Renaissance period, Italian woodworkers were famous for selecting wood for artistry and they were highly skilled when working with grain patterns and coloration. The range of wood colors varied from locally sourced light-colored wood to dark wood such as oak obtained from bog areas. Intarsia, a form of wood inlaying, often utilized spalted wood (Blanchette et al., 1992; Michaelsen, 1992). The most common spalted wood used for intarsia was green colored (from *Chlorociboria* species), and its use was quite outstanding in various works. The green-coloration is evident in the works of da Veron, who lived between 1457 and 1525, and the works of da Maiano who lived between 1432 and 1490 (Blanchette et al., 1992; Michaelsen, 1992).

The practice of using green-colored wood along with other types of spalting is reported to have been passed on from place to place, even as far as the Italian Alps. The 16th century saw the spread of intarsia throughout Europe, from Switzerland to the south of Germany and with it, the spread of spalted woods. During this period, green stained wood was popular because it signified the Alps to the many German-speaking people in Europe. In Switzerland, the use of blue-green pigmented wood is exhibited in the wood-paneled veduta that is preserved in the Museum of Decorative Arts in Berlin. The same museum has a green colored Tyrolean wall that shows the use of color from *Chlorociboria*. Green colorations were also evident in wood used in door and wall panels in the room of Schloss Velthurns (Ricchebuono, 2011). The green stain was used in palaces and castles to produce characteristic green leaves, and to represent vegetation on wall and door panels.

Most important was the knowledge that the pigmentation was long lasting. In addition, it exhibited some luminous characteristics. A famous use of the wood is in Wrangelshrank cabinets, which exhibit a rich use of intarsia throughout their design. Spalting is also shown, with green pigments used for trees, leaves, landscape, and grass on its exterior, and green pigmentation on the interior (Lorenz, & Jutzi, 2011).

In the 17th century, there was an increase in the use of dyeing and staining techniques based on knowledge obtained from the 16th century. During this century, spalting was evident throughout Franconia, northern Germany, and Augsburg where the blue-green staining by *Chlorociboria* was most noticeable (Nielius, 2002). It was also evident in Bohemia where green colored patterns were used.

Throughout the 18th century, green wood spalted with *Chlorociboria* was used, in addition to zone lines and white rot, as an element in design, in the veneer of walnut.

The 19th century was a remarkable period due to industrialization that saw marquetry and veneer techniques used as never before. As a result of industrialization, there were improvements in dyeing and staining techniques, which led to an overall decrease in the use of spalted wood (Michaelsen, & Buchholz, 2009). The one remaining area of use was in Tunbridge Wells, England, where the famous Tunbridgeware was produced using *Chlorociboria* wood for the blue-green coloration. Also in this century, the fungal species *Fistulina hepatica*, which produced a red-brown coloration, was widely researched as were zone-lines (Willkomm, 1866).

In the 20th century, the industrial revolution turned consumers completely away from spalting. However, spalting was rejuvenated when the need arose for materials that had received minimal manufacturing. Most importantly, the Arts and Crafts movement played a critical role in its rejuvenation because it stressed the use of natural, hand-worked designs over those created *en mass*.

In 1970, spalting once again became a mainstream art form. Mark Lindquist, a noted woodturner, successfully brought zone line spalting into fashion in woodworks (Lindquist, 1977), and the use of spalted wood in art and craft has experienced a growing resurgence since this time.

To date, the USA has been the primary developer of spalting, and research is continuing (Croan, 2000; Beakler, 2012; Beakler, 2013; Robinson, 2007-a; Robinson, 2007-b ; Robinson, 2009; Robinson, 2012-a; Robinson, 2013-a; Robinson 2014-a).

Although there has been a huge shift in consumer preference into character wood for decorative purposes in the last century (Nicholls 2002; Donovan and Nicholls, 2003), spalted wood is still considered rare (Loyalist Forest Products Inc. 2009; Bell Forest Products, 2010). The recent customer demands and interests in spalted wood has led to a continuing demand for research and methodology to reproduce the pigments for commercial use (Phillips 1987; Robinson et al. 2007-a; Robinson et al., 2010-b). At the beginning of the 21st century, spalting research mostly focused on the induction of melanized zone line-type spalting and only slightly to the colored fungal pigments (Robinson, 2012-a). Studies also focused on production of zone lines and pigmentation on wood using live fungal culture inoculations techniques (Robinson, 2007-b).

Spalting stimulation research discovered that many fungal species such as *Xylaria polymorpha* can produce zone lines or black pigment when grown under specific sublethal conditions such as medium containing copper sulfate (Robinson et al., 2010-a). *Scytalidium cuboideum*, which produces red pigment on sugar maple (*Acer saccharum*), is also capable of producing blue stain in the presence of copper sulfate (Robinson et al., 2010-a).

In 2014, research turned from inoculation with live fungal cultures to pigment extraction. Spalting pigments were extracted with different organic solvents. Dichloromethane was found to be the most effective solvent (Robinson, 2014-a). Another study investigated the surface pigmentation of different wood samples by extracted fungal pigments and application using pressure treatment (Robinson, 2014-b). This study was very successful for quick surface pigmentation and did not produce any visible internal pigmentation (Robinson, 2014-b). The results of these most recent studies promises a strong future for the use of extracted pigments in the dye industry.

1.5 Spalting Fungi

1.5.1 *Chlorociboria* Species

Chlorociboria belongs to the subdivision pezizomycetes with the division Ascomycota, class Leotiomycetes, and the family Helotiaceae. This genus of fungi contains 17 species including *Chlorociboria aeruginosa* and *Chlorociboria aeruginascens.* These two species can be distinguished by observing their microscopic structures. They differ especially in the size of their ascospores. *Chlorociboria* has a worldwide distribution and can be found on well-decayed wood. Wood colonized by *Chlorociboria* species appears blue–green due to the extracellular pigment xylindein produced by the fungus. Fruiting bodies of *Chlorociboria* are more likely to appear in the fall after a rain. The fruiting bodies are initially cup-shaped and later become flattened or disc shaped with a length of up to one centimeter. The fruiting bodies have a tiny stem, which may be placed centrally or off-center (Blanchette, 1992; Gümbel, 1858; Rommier, 1868; Blackburn, 1963; Saikawa, 2000).

Chlorociboria species have shown a preference for some tree species over others (Robinson et al., 2012-b; Robinson, 2012-c). Generally, *Chlorociboria* species are not considered decay fungi. They do not degrade the cell walls directly but instead colonize wood that has already been decayed by other types of fungi. They are, therefore, regarded as secondary colonizers.

1.5.2 *Chlorociboria aeruginosa*

This species of *Chlorociboria* (formerly *Chlorosplenium aeruginosum*) is found predominantly on decaying wood of trees from the genus *Populus*. *Chlorociboria aeruginosa* produces larger spores than *C. aeruginascens*, which are between 9–15 µm length 1.5–2.5 µm widths. It has round elongated cells and spores measuring between 6–10 µm × 1.5–2 µm. This species has a tomentum, which is located in the upper part and is very delicate, consisting of a smooth, round elongated structure (Tracy, 2002). The fruiting bodies commonly occur in fall and summer and are widely distributed in North America. *Chlorociboria aeruginosa* has hyaline spindle- to ellipsoidal-

shaped spores with oil droplets at each end. The hyphae grow in ray parenchyma within the wood, and the hyphal mycelium are visible under a light microscope. A cup-shaped fruiting body forms when the moisture and relative humidity of the substrate are high (Tracy, 2002).

1.5.3 *Chlorociboria aeruginosa* in the Laboratory

Factors that affect the growth and germination of *C. aeruginosa* are high levels of sucrose and glucose, which may stimulate their effects on wood (Robinson, 2011-a, 2012-b). In laboratory cultures, *C. aeruginosa* may stop producing xylindein, although the cause is not known. If xylindein is not produced, cultures continue generating white mycelium even after being re-plated onto fresh media.

1.5.4 *Chlorociboria aeruginascens*

This species is very similar to *C. aeruginosa,* with the primary difference being the smaller ascospores (5–7 µm length × 1–2 µm width). *Chlorociboria aeruginascens* is distributed within temperate forests throughout the world.

The synonym of *C. aeruginascens* is *Peziza aeruginascens.* It is derived from the Latin words aerug which means “blue-green” and ascens which means “becoming”. *Chlorociboria aeruginascens* creates small, saucer-shaped fruiting bodies and produces extracellular xylindein in the colonized wood. It has apothecia that are 0.5-cm diameter, hooked laterally. These structures collapse and roll inwards when dry (Ramamurthi, 1957*).* It has smooth and delicate hyphae that are hair-like and tomentose. The stipes of the fruiting bodies are 3-mm long with an eccentric attachment to the apothecia. The spores are rough and spindle shaped. It can be difficult to differentiate between *C. aeruginascens* and the larger varieties of the genus, although this species is distinguished by its smooth tomentsum hyphae whereas *C. aeruginosa* has rough hyphae (Ramamurthi, 1957*).*

1.5.5 Coloring of Wood by *Chlorociboria* spp

Coloring caused by *C. aeruginosa* and *C. aeruginascens* is due to production of the pigment xylindein. Chemists classify this pigment as a napthaquinone. The history of this pigment dates back to the year 1728 when it was known to be a pigment that caused coloration in wood. In 1874, Liebermann demonstrated that it was a quinone. Liebermann used aqueous phenol to obtain xylindein from wood infested with fungi and then crystallized it (Liebermann, 1874). Studies of the pigment indicated that felled wood showed signs of pigment penetration but did not show any signs of texture change.

Blackburn (1963) was of the opinion that to evaluate its structure, it was vital to identify the functional groups that comprise the molecule and to ascertain the molecule's central skeleton. He noted that a structural evaluation was significant because it helped to comprehend how the molecule could be chemically altered. Blackburn (1963) discovered that xylindein's chromophore was similar to that of numerous artificial dyestuffs.

The mass of the molecular ion and the molecular structure has been determined using mass spectrometry (Blackburn, 1965). Xylindein has been found in abundance in many other homologous compounds. Saikawa et al., (2000) used X-ray crystallography to determine xylindein's configuration and found that it was composed of two similar parts developed from eight two-carbon components (Blackburn, 1965). One disadvantage of this pigment is that it inhibits plant germination, which has led to its evaluation as an algaecide (Rai, 2009).

1.5.6 *Scytalidium cuboideum*

The fungus is classified in the phylum Ascomycota, the class Pezizomycotina, order Leotiomycetes, genus *Scytalidium*, and species *cuboideum*. There are three common strains denoted by the numbers CBS 241.61^{T}, CBS 192.80, and CBS 409.84 (Global Catalogue of Microorganisms). It grows throughout North America in many forest types. It attacks both hardwoods and softwoods. It

does not significantly affect the strength of the wood, but it can cause soft rot. The mycelium of *S. cuboideum* produces an extracellular pigment in colonized wood, often a red/pink color but sometimes a tyrian blue (Golinski et al., 1995, Kang et al. 2010). This species has also been linked to infected logs utilized in shiitake mushroom farming.

The pigment produced by this species of fungus is thought to be a derivative of a naphthoquinone (Unger, 2001). The fungus can completely colonize and color sugar maple within ten weeks after inoculation (Robinson, 2011-a). The pigment has high potential as a dye in the textile industry (Weber et al., 2014). Recent research shows that the fungus has been found in human samples from clinics in the US, which were identified by molecular techniques and morphology (Giraldo et al., 2013).

1.5.7 *Scytalidium ganodermophthorum*

This fungus produces penetrating yellow pigments in colonized wood. It is classified as phylum Ascomycota, class Pezizomycotina, order Leotiomycetes, family Leotimycetidae, and genus *Sytalidium*. The yellow pigments have been studied by researchers such as Kang et al. (2010) who found this species has a characteristic anamorph that is arthroconidia-like. The pigment is described as being very destructive. Under laboratory incubation, the fungus takes around 12 weeks to completely color sugar maple wood (14-mm cubes). This particular fungus displays antifungal activity. It contains an active ingredient that has been found to inhibit pathogens of plant growth (Schowanek et al., 2001). Furthermore, Robinson et al. (2014-a) demonstrated that *S. ganodermophthorum* produced a yellow pigment that penetrated non- sterile logs within 12 weeks.

1.6 Significance of the Toxicity of Fungal Pigments

Many fungi produce metabolites that are known to have toxic effects. Fungal toxins are called mycotoxins, which include the pigments. Mycotoxins are secondary metabolites that have toxic effects on humans and animals and cause mycotoxicosis. The type of mycotoxin determines the

impact on the animal. Other factors that might contribute to the extent of mycotoxicosis include the age of the animal, nutritional status, exposure, and the chemical structure of the mycotoxin. Interest in mycotoxins arose in 1960 when a mycotoxicosis emerged that caused turkey X disease in farm animals in England (Wolstenholme & Cameron, 1954). The cause of the disease was an aflatoxin. An outbreak of ergotism in Europe reached epidemic proportions in the Middle Ages, killing thousands of people. It was caused by alkaloids produced by *Claviceps purpurea.* Such effects make it necessary to study the potential toxicity of fungal pigments to ensure that use of these pigments does not cause harm. Acute mycotoxicosis can be a serious and sometimes fatal disease. Most mycotoxicoses have been caused by ingestion of food contaminated with fungal pigments. It is, therefore, advisable to study the toxic effects of such compounds on human and animal health, including the pigments produced by fungi (Blaise et al., 1988).

1.7 Toxicology

1.7.1 Stages of Zebrafish Development (life cycle):

According to Nusslein-Volhard & Dahm (2002), zebrafish grow to a length of four to five centimeters throughout their lifespan of two to three years. Adulthood begins 90 days after fertilization with an initial average length of 2-3cm. Like other fish, the zebrafish passes through larval and juvenile stages to grow to adults. However, Kimmel et al. (1995) noted that the process of development in zebrafish species is not well known compared to other organisms. The stages of development in zebrafish are named rather than numbered to allow for flexibility for adjustments as more is still being learned about the species. Usually, the basis of staging and naming is the morphology of the organism that is obtained by examination of the live embryo.

The embryogenesis of *Danio rerio* involves seven stages as defined by Kimmel et al. (1995). It begins with the zygotic period through cleavage, blastula, gastrula, segmentation, pharyngula, and hatching phases. The stages of embryonic development that bring about the morphogenesis of the zebrafish happen in the first three days after fertilization.

The first phase of early development is the formation of a zygote. It is formed immediately after fertilization when a newly fertilized egg goes through cell division to the end of the zygotic cell cycle one during a period of 45 minutes post-fertilization (Schier, 2005). At the end of the three-quarter hour period, the next phase called cleavage begins. Here, two to seven cell cycles occur rapidly and synchronously for up to 2.25hrs. It is followed by the blastula period that gives rise to eight and nine cell cycles. The process is rapid and metasynchronous. There is also a transition period known as midblastula marked by a prolonged asynchronous cycle and the beginning of epiboly formation before progression to the next stage of development. After 5.25 hours of fertilization, the gastrula phase begins. It is characterized by developmental movements including contraction, convergence, involution as well as the extension of the epiboly end to form the epiblast, hypoblast, and the embryonic axis (Solnica-Krezel, 2006). This stage runs up to 10 hours when segmentation begins. At this point, there is the development of the pharyngeal arch primordia, somites, and neuromeres. Furthermore, primary organogenesis occurs, the tail appears, and earliest movements are observed (Harvey, 1999). The pharyngeal period happens 24-hour post fertilization, and it is the phylotypic stage of the embryo development, with the body axis straightening from its initial curvature about the yolk sac. The circulation, coloration, and fins start to develop. Larvae hatch after 48 hours. It is asynchronous, and the rapid morphogenesis of primary organ systems, cartilage development in both head and pectoral fin observed in earlier stages ceases. After 72 hours of fertilization, the early larva is formed with a well-developed swim bladder that is inflated. The zebrafish at this stage show characteristic behaviors of active avoidance and food seeking. The larvae then grow to juveniles that develop to adults after about three months post fertilization of the eggs (Isogai, 2001).

1.7.2 Zebrafish as an Animal Model for Toxicology and Other Applied Research

There are a variety of lab animals used either as *in vitro* or *in vivo* models for studying toxicity of colorant solutions. The zebrafish (*Danio rerio*) is a tropical freshwater fish that has been well characterized (Kimmel, 1995; Haffter, 1996). Subsequently, zebrafish are a widely studied model organism for toxicology and genetics as well as developmental biology (Detrich, 2010; Kari, 2007; Ellis, 2014; Zhang, 2015; Kar, 2015). George Streisinger pioneered the use of zebrafish as a laboratory animal while at the University of Oregon with his colleagues (Stahl, 1995).

Zebrafish are extensively used for research, understanding developmental mechanisms, and disease processes. The extensive use of zebrafish is attributed to their ability to breed readily. Zebrafish are capable of laying hundreds of thousands of embryos weekly (Kari, 2007). Moreover, their small size (3-5cm) allows for easy handling and storage in shoals. Zebrafish embryos are transparent and can be easily observed for morphological changes. The embryos are also easily manipulated genetically. Use of zebrafish as a tool for understanding human diseases has been in existence for the past fifteen years (Barut, 2000) and therefore considered a great model for studying human diseases (Zon, 1999). They are used for analysis of vertebrate development due to their close homology with humans (Driever, 1996).

The zebrafish model is a well-validated model that can explain the biological function through their genetic analysis (Chan, 2002; JR, 2003). Zebrafish assays are extensively used to assess bioactive drugs and therapeutic compounds for pharmaceutical applications (Zon, 2005; Bowman, 2010). Toxicological research initially concentrated on using zebrafish models for ecotoxicology and acute toxicity testing although advancements have been made in the field of toxicology (Nagel, 2002). The zebrafish model offers an opportunity to understand the mechanisms beyond acute toxicity (Strähle, 2012) as advanced toxicological studies are used to study causes of human toxicity and teratology (Redfern, 2008; Eimon, 2009). Some *in vivo* assays that were based on zebrafish embryos and larvae were established to examine the toxicity of chemical compounds in the environment

(Yang, 2009). Also, the assays were used to screen for potential drug toxicity during early stages of drug discovery (McGrath, 2008; Eimon, 2009).

Zebrafish can also be used for environmental monitoring to evaluate organic pollutants, toxic metals and endocrine receptors (Mizell, 1997; Busquet, 2008). Therefore, they are useful in assessing the impact of pollutants that are constantly released into the environment. They are also used as a model to investigate the potency of natural products from plants and other organisms such as secondary metabolite extracts (Crawford, 2011; Challal, 2012).

1.7.3 Characteristics and Disadvantages of the Zebrafish Model

There are many advantages of using the zebrafish model for the study of toxicology. The popularity of using the zebrafish embryo increased as an alternate animal model for hazard testing and risk assessment (Embry, 2010). Also, the use of zebrafish embryos is not restricted by animal welfare regulations such as the revised European directive 86/609/EEC of September 2010 (European Commission, 2010). A lot is known about the biochemistry, morphology, and physiology of all stages of development of juvenile and adult zebrafish of both sexes (Detrich, 1998). Zebrafish are similar in cellular structure, anatomy, signaling processes, and physiology to other higher-order vertebrates. Zebrafish development resembles that of higher vertebrates since they grow and develop outside the mother (Mathias, 2012). Recent studies on zebrafish have focused on developmental biology. Detecting abnormalities in the lab has facilitated identification of many human diseases including cancer (Rubinstein, 2003; Amatruda, 2002; Brittijn, 2009). The embryos develop rapidly compared to mammals and show defined morphology such as cardiac muscle, liver, gonads, somites, swim bladder, muscular tissue, yolk, axis and tail (Kirschbaum, 1975; Schmitz, 1994; Schilling, 1997; Iovine, 2000; Isogai, 2001; Robertson et al., 2007; Patterson et al., 2008). The transparency of the embryo allows visualization of organ systems and any abnormal changes (Brittijn, 2009; Muncke, 2006).

The small size of the zebrafish allows maintaining large numbers of these vertebrates in reasonably small spaces, minimizing the costs incurred in husbandry. Their size also minimizes the quantities and costs of the test substances required including chemicals and environmental pollutants (Ghiassi, 2014). In addition, the optimum breeding and maintenance conditions in the laboratory are easy to maintain. The female lays around 200 eggs per week, which enables the study of large numbers of embryos for potential toxicity (Dahm, 2006; Brittijn, 2009). There are many studies focused on the relationship between the zebrafish and human genomes. The studies have enhanced identification of the roles of human genes from zebrafish mutations. Through studies based on zebrafish model, genes mutations such as orthologues that cause human diseases have been identified. (Brownlie, 1998). The zebrafish genomic sequence was completely mapped in 2009 (Bhartiya, 2010). The mapped zebrafish genome facilitated understanding the homology and function relationship between zebrafish and human genes including non-coding regions (Shin, 2005). Zebrafish have unusual gene similarity to humans, which makes the genes an important model (Howe, 2013). About 71.4% of human genes have at least one zebrafish orthologues while 69% of zebrafish genes have at least one human orthologues (Howe, 2013). Eighty four percent of zebrafish genes are equivalent to human genes that are associated with diseases (Rodríguez, 2011).

It is also possible to introduce genetic changes into the zebrafish embryos (Streisinger, 1981). The zebrafish as a vertebrate has major organs that share many features and functions with humans such as kidney, liver, pancreas, muscles and blood (Wallace, 2005). These reasons make the zebrafish model useful as a candidate for chemicals screening, as well as other applied research such as toxicology and teratology (Kari, 2007). Although there are advantages of using zebrafish in studies related to human toxicity, there is a clear divergence between human and zebrafish in form and function. For instance, zebrafish do not have some features possessed by human such as synovial joints, hair follicles, lungs, limbs and bone marrow (Jagadeeswaran, 1999; Gray, 2009; Brittijn, 2009). Contrary to human normal physiological temperature, zebrafish are cold-blooded vertebrates whose embryos require a temperature of 28.5°C for raising (Haldi, 2006). The temperature difference leads

to differences in response after exposure to a similar agent. Therefore, it will be difficult to administer or study toxicity of a small, poorly water-soluble agent, on zebrafish without a carrier solvent such as dimethyl sulfoxide (Goldsmith, 2012). The zebrafish model also lacks a standard diet (Kimmel, 1995). Another important consideration is development. Zebrafish have undergone an extra round of partial genome duplication, which means that 20% of mammalian genes have two orthologues in zebrafish (Amores et al., 1998; Dahm, 2006). Duplications of genes may change the gene function of at least one member of the gene pair (Prince, 2002). There is increasing certainty and developing knowledge about comparative genomes, anatomy and physiology of zebrafish to that of humans. Therefore, there is more application of the vertebrate model. Still, the known unique properties of the zebrafish model makes it a powerful *in vivo* system for studying toxicity and modeling human disease.

1.7.4 Zebrafish Embryo *in Vivo* Acute Toxicity Assay Using 96-well Plates and the Components

Their rapid development, short generation time and short life cycle make the zebrafish very useful in the evaluation of toxicity of environmental chemicals. The toxicity testing is useful in determining compound doses as well as identifying responses to toxicity. The *in vivo* acute toxicity of the zebrafish embryo is one of the methods that can be applied (Brannen, 2010). In this method, plates with 96 wells contain the developing embryo and the solution for analysis is used. Delvecchio et al. (2011) noted that the exposures are done from 8 to 120 hours after fertilization because it is the period the organism still obtains its nutrient from the yolk sac. When using the 96-well plates, small volumes of solution are required for exposures and the transparency of the embryo allow for the non-invasive evaluation of morphological, developmental and behavioral characteristics (Murphey & Zon, 2006).

The adult species are reared in standard laboratory conditions of 28°C with a pH of about 7-7.2 (Peterson, 2012). Adequate feeding is provided as well as maintained water circulation. Spawning is done when embryos are needed, and the fertilized eggs collected in fish water in a Petri dish before being put in an incubator six hours post fertilization. After the dechorination, waterborne exposure

can be done by dissolving the chemicals in fish water or adding dimethyl sulfoxide (DMSO). The chemical solution is placed in the wells of the multi-well plate. Two plates containing the concentrate solution and the control are used where the viable developing embryos are transferred into individual wells at 8-hour post fertilization using a wide-bore glass pipette (Truong, 2011). Finally, assessment of viability, development, and spontaneous movement is done after 24 hours. The morphological and behavioral endpoints for the brain, heart, eye, body axis, swim bladder and motility, and tactile response are determined at 120hpf. The components of this assay method include zebrafish husbandry, dechorination, exposure, and assessment. According to McGrath, & Li (2008), during husbandry, the embryos are incubated in water in preparation for investigations. It is followed by removal of the chorion by a pronase enzyme to eliminate barrier posed by it to the solution, a process known as dechorination (Kim, & Nelson, 2006). It allows viewing of the embryos with a compound stereo-microscope, and it was done in a 60 mm glass petri dish containing 50 mg/mL pronase (Henn &Braunbeck, 2011). The dechorinated embryos were then exposed to the chemical solution in the 96-well plate using a 8 or 12 multi-channel pipette, reagent reservoir, and wide-bore Pasteur pipette. The final component is an assessment that requires anesthesia with a solution of 3-aminobenzoate ethyl ester methanesulfonate salt and methyl cellulose.

1.7.5 Research Hypothesis

The hypothesis of this study was that the acute toxicity of pigments extracted from four species of fungi could be determined via their adverse effects on zebrafish embryos. This study measured acute toxicity on embryos at 120 hours post fertilization by observing any abnormal growth following 24 hours of exposure to 100%, 200%, or 400% concentrations of each pigment. Conclusions were then drawn as to the toxicity of these pigment solutions in relation to the zebrafish embryos.

1.7.6 Analysis

Evaluation was in binary notation (toxicity present or not). The study focused on the relationship between pigment mixture and abnormal response in the fish embryos. The questions of interest were:

A) Were there any abnormalities following the exposure to each pigment mixture from DCM extracts and a purified material?
B) Were there any abnormalities following the exposure to each mixture from the malt liquid culture pigment solution?

A Fischer exact test model was used to study the relation of pigment mixture and response (Troung et al., 2011). This test used to evaluate data in binary notation (present of the responses or not present). It is statistical comparison between the control and pigment solutions exposed embryos at $p<0.05$ for each evaluated endpoint (mortality, delay in progressions, and deformations). The experiment is considered not valid if two embryos die in the control group, at which point the experiment should be repeated.

1.7.7 Relevance of Study

Interest in reproducing natural spalting pigments has a long history and the use of these pigments is increasing worldwide. The extracted pigments from spalting fungi are a potential alternative to synthetic dyes that can be highly toxic in nature, have adverse effects on both human health and the ecosystem, and are generally made from nonrenewable materials. The goal of the research was to examine any adverse effects that could be produced by extracted fungal pigments from a select group of spalting fungi. If these pigments are to be used commercially, it is important to understand their potential toxicity to humans and animals.

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2.0 Methodology

2.1 Fungal Growth

Spalting fungi (*Chlorociboria aeruginosa, Chlorociboria aeruginosa, Scytalidium cuboideum,* and *Scytalidium ganodermophthorum*) were selected for testing because of their high production of extracellular pigment and their use in previous spalting research. *Chlorociboria aeruginosa* UAMH 11657 was isolated from a decaying hardwood log in Haliburton, ON, Canada, *Chlorociboria aeruginascens* UAMH 7615, was isolated in Lake District, UK, *Scytalidium cuboideum* (Sacc. & Ellis) Sigler UAMH 11517, was isolated from *Quercus* sp. in Memphis, TN, USA, and *Scytalidium ganodermophthorum* UAMH 10320 was isolated from oak wood logs in Gyeonggi Province, South Korea.

Fungal isolates were grown and fungal growth was determined using agar-based and the liquid culture method as outlined by Robinson et al. (2012-a; 2014-b). The agar-based method mixes finely ground spalted wood chips into 2% malt agar as a growing medium for pigmenting fungi. The inclusion of these wood chips stimulates pigment production, especially in *Chlorociboria* species (Robinson et al., 2012-a). The liquid culture method used fungi inoculated onto 2% malt extract in pint Mason jars. All cultures were then incubated at room temperature (21 degree Celsius) for 28 days on an open shelf. Testing as detailed below was done with pigments suspended in a DCM solution (agar-based), and pigments suspended in the malt water solution (liquid culture), to compare toxicity between these two distinct growing methods.

2.1.1 Organic Solvent Extraction

Dichloromethane (DCM) was used for pigment extraction due to its rapid diffusion through various materials, including wood blocks (Robinson et al., 2014-b). Cultures were prepared for extraction in one of two ways. The agar plates were dried for two days. The resulting wood mat was then shredded into roughly 5mm pieces and placed in a 250 mL Erlenmeyer flask. Forty-five mL of

dichloromethane was added to the Erlenmeyer flask, along with a 2x5 mm magnetic stir bar. The 250 ml flask was sealed, placed on a Dylastir stir plate and rotated at 220 rpm for 30 minutes. The resulting solution was then filtered through watman filter paper to remove large particulate. The extract was collected in a borosilicate glass vial and sealed with non-evaporative polyseal-cone-lined caps. About 3 mL of the solution was added to a glass cuvette for analysis on a Konika-Minolta Chroma meter CR-5 colorimeter. After reading by colorimeter, the dye solution was returned to the vial and stored for future use (Robinson, 2014-a). Liquid cultures were not extracted, and were used directly from the flask.

2.1.2 SPE Column Extraction and Purification

This method was used for extraction-purification of pigment to reduce the amount of undesired chemical contaminants before the solution was exposed to the zebrafish embryos. The method is currently under continual development (Remcho, unpublished, Linus Pauling Center, OSU Corvallis).

The liquid malt medium inoculated with *C. aeruginosa*, *S. cuboideum*, and *S. ganodermophthorum* was extracted per the methods outlined above. A Strata SPE 2g/12mL column (Phenomenex Company) was conditioned by adding 4 ml of acetonitrile solvent to remove trapped air and activate the SPE particles. The solvent was removed and the sorbent interaction with target analytes was maximizing by adding 4ml HPLC grade water. The sample of pigment solution was loaded onto the column and then washed by adding 10 ml of acetonitrile and HPLC grade water mixture to remove contaminants that were not bound to the sorbent. The final step to elute the target pigment was to add 2-4mL 100% EMD-HPLC chloroform.

About 10ml of the pigment mixture sample was used to achieve less than 0.5 ml purified pigment in two to three hours.

2.2 Determining Concentration

Standards have been developed to determine pigment concentration based on L*a*b* values, not dry weight, when working with agar-plate fungi (Robinson et al., *in review* a). The color values are used instead of the dry weight because of the range of color saturation and hues produced even under the same conditions and with the same species. Simple dry weight cannot be used for estimating concentration because different colors may exist within the same weight of pigment. Instead, solution concentration was standardized to +/- 2 for the L*a*b* values determined to be the 'standard' for these fungi. The standard L*a*b* values referenced in Robinson et. al. 2014b, are: for *C. aeruginosa* L* = 82.28, a* = −11.06, b* = −5.40; *S. cuboideum* L* = 82.32, a* = 26.84, b* = 13.19; *S. ganodermophthorum* L* = 95.46, a* = −3.00, b* = 8.15.

For color values from the liquid cultures, the concentration was determined by taking readings from the liquid cultures once incubation was concluded, and dividing the L*a*b* values by the laboratory standards. The liquid culture pigment concentrations could not be changed due to the low water solubility and high aggregation property of the pigment. This leads to strong adherence of these pigments to solid surfaces. The amount of pigment produced by the fungus reaches the level of aggregation after two weeks of incubation; hence the addition of more pigment in attempts to create higher concentrations would cause the pigment to precipitate.

2.3 Preparation of the Zebrafish

The zebrafish embryos were obtained from a Tropical 5D strain of zebrafish (*Danio rerio*) raised in the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University, Corvallis. The preparations and experiment were performed in following Good Laboratory Practices. Adult zebrafish (*Danio Rerio*) were maintained under appropriate conditions of a diurnal period of 10 hours dark and 14 hours light at 28 C and pH 7 ± 0.2. Water environments suitable for them to thrive were prepared in the laboratory. The adult zebrafish husbandry was described by Troung et.al. (2011).

After fertilization, the eggs were collected in a petri dish. An Olympus-SZ51 stereomicroscope was used to identify abnormal and non-fertilized eggs which were removed (Ali, 2011; Troung, 2011). The sorted normal fertilized zebrafish eggs were in very late blastula stage (4-5 hpf) (Hisaoka, 1958), which corresponded to 2.5-3 hpf (Kimmel *et al.*, 1995). The fertilized eggs with the same quality were used.

2.4 Zebrafish Testing Assessment – General Overview

The zebrafish test method used to assess toxicity was modified from the Troung method (Truong et al., 2011). The procedural steps are zebrafish dechorination, exposure and assessment. After six hours post-fertilization, all normal embryos were dechorionated following a modified Wester field's protocol (Truong et al., 2011). After 6 hpf, the embryos were placed into a 60 mm glass petri dish with 25 mL fish water and exposed to 50 µl of 50 mg/mL pronase enzyme to degrade the outer chorionic layer. Under the microscope, the Petri dish was gently rotated to mix the solution while observing the separation of the embryos chorions. After chorion deflation ~7 minutes, the solution was diluted with fresh fish water. The diluted solution was the poured slowly over the edge of petri dish into the sink. The dilution was repeated for 10 minutes. The dechorionated embryos were recovered in the Petri dishes at room temperature until 8 hpf.

The exposure procedure was conducted by waterborne exposure. A hundred microliter of water and 100 µL of each pigment treatment were added into each well. At eight hours post fertilization, each embryo was then placed in its own well in a 96–well plate. The embryos were incubated at 28°C for 24 hours post fertilization for the first assessment (Truong et al., 2011).

The first assessment was undertaken at 24 hours post fertilization. The embryos were evaluated for advancement of development, impulsive movement, and viability. At five days post fertilization, the morphology of the larvae was assessed, which constitutes the jaw, body axis, snout, pigment, circulation, yolk sac, fin, eye, and swim bladder (Truong, et al., 2011). The behavioral endpoints were also assessed (Truong, et al., 2011). The assessments were conducted in a binary

form, as present or not present. The Fisher's exact test at $p < 0.05$ was used to statistically evaluate the pigment exposed and the control groups (Truong, et al., 2011). Toxicity of pigments on zebrafish embryos was tested at 100%, 200% and 400% of the standard L*a*b* values for agar wood and liquid malt cultures.

2.5 Applying Pigment to Well Plates

2.5.1 Agar Wood-DCM Solution

For each assigned concentration of DCM pigment extract, 100 µl of each pigment extract was placed in four separate Zinser 96-well glass petri dishes and the solvent was allowed to evaporate under a fume hood for 24 hours or until the DCM had evaporated completely. After evaporation, 200 µl of a fish water containing 0.3g/L of aquatic salt was placed in the Zinser 96-well glass petri dish (Troung et.al 2011). The fertilized eggs (about 200 eggs) were collected and rinsed several times in water and 50 µl of 50 mg/mL of pronase enzyme (Sigma-Aldrich, cat # 81750), which was used to remove the egg outer layer (chorion). Then the eggs were placed into 200 µl fresh fish water in the Zinser 96-well glass petri dish. The Zinser 96-well Petri dishes were incubated at 28°C until 24 hour post fertilization hpf, then the appropriate assessments were performed (Troung et.al, 2011).

Three different doses of each pigment extract were added to four separate Zinser 96-well glass petri dishes that contained the fish water. Next, one dechorionated zebrafish embryo was added to each well. The Petri dishes were incubated with the waterborne exposed zebrafish embryos at 28C until 24 hpf and then an assessment was performed. Following the previously described methodology, four experiments were run to screen toxicity of the DCM pigment extracts.

2.5.1.1 Experiment 1. Wood Agar at 100% Concentration, First Run (all pigment run on the same 96-well plate):

Between 12 to 24 zebrafish embryos were used for each pigment extract. Two different

controls were used, the fish water or dichloromethane (applied in the well then allowed to evaporate) (figure 1).

2.5.1.2 Experiment 2. Wood Agar at 100% Concentration, Second Run (each pigment was done in its own well plate):

In experiment 2, the 100% concentration pigment tests were repeated using 72 embryos for each pigment extract. The same two controls were used (figure 2). Experiment 2 only differed from experiment 1 in terms of number of replicates, and was performed to verify the findings from experiment 1.

2.5.1.3 Experiment 3. Wood Agar at 200% Concentration (all dyes in same well, different numbers of controls and treatments than before):

In experiment 3, a 200% concentration extract was prepared and applied on 72 embryos for each pigment extract. The same controls were used as in experiment (figure 2).

2.5.1.4 Experiment 4. Wood Agar at 400% Concentration (same set up as experiment 3):

In experiment 4, a 400% concentration was prepared and applied to 72 embryos for each pigment extract. The same controls were used as in experiment 1 (figure 2).

2.5.2 Liquid Cultures

Three categorized solutions (live, autoclaved and filtered) were tested from each fungal species. For each solution, 100 micro-liters of pigment solution was transferred into a Falcon sterile 96-well plate with a working volume 1.5 to 2.2ml and dissolved in fish water. Only one concentration of categorized pigment solution and two controls group can be tested for each liquid culture media pigment using 96-well plate each time due to size limitations. The two controls were fresh fish water and sterile liquid malt extract.

At eight hpf, one viable, appropriately developing embryo was transferred into each

individual well of a 96-well plate using a disposable wide-bore glass pipette. The plates were incubated at 28°C until 24 hpf, then assessed (Troung et al., 2011). The number of embryos tested in each 96-well plate included 24 controls and 12 to 24 embryos for each pigment solution. Following the previous descriptive method, five experiments were run to screen toxicity of each pigment solution.

2.5.2.1 Experiment 5. Liquid Malt (live culture-all with every color pigment solution on the same well plate)

In experiment 5, a direct pigment solution from live cultures from malt liquid media was applied on 12 embryos for each pigment solution (figure 3).

2.5.2.2 Experiments 6. Autoclaved Liquid Malt (all dyes on same well):

Each pigment solution from each fungal species was autoclaved at 120 C for 30 minutes. The autoclaved pigment solution from the malt liquid culture media was applied on 12 embryos for each pigment solution (figure 3).

2.5.2.3 Experiments 7. Filtered Liquid Malt (all pigment solutions on same 96-well):

In experiment 7, the pigment solutions were filtered to remove fungal cells from medium using 0.2 micron EMD Millipore filters. Each filtered pigment solution was applied to 24 embryos (figure 4).

2.5.3 Purified Pigment Solutions Using SPE Column and HPLC Technology

Purified pigments were assessed using the SPE column method (Remcho unpublished). The liquid malt medium on which *Chlorociboria aeruginosa, Chlorociboria aeruginascens* and *Scytalidium cuboideum* were cultured, were purified using Strata SPE 2g/12mL columns (Phenomenex Company) using procedures described in section 2.3.

Each purified pigment was evaporated under sterile conditions in a fume hood overnight. The pigment was then washed with HPLC grade EMD methanol solvent and centrifuged at 12000 rpm for 10 minutes. Finally, the precipitated pigment was collected and dissolved in acetonitrile for analysis by HPLC on a SHMADZU-USA model HPLC. The purified pigment was analyzed with mobile phases 30-95% concentration HPLC grade acetonitrile in HPLC water. A phenyl bonded reverse phase column for aromatic compounds was used for pigment analysis. The software method was developed by the Remcho analytical chemistry laboratory. Each sample run consisted of 30% acetonitrile for first three minutes (0 to 3 minutes), and for the gradients 30-95% acetonitrile from 3 to 13 minutes to flush and clean the column of any contaminants. Afterwards, the column was brought back to 30% acetonitrile to equilibrate for the next run. Each sample took 25.5 minutes to run. The run started when the HPLC oven temperature hit 40 C degree. Acetonitrile control samples were run before and after each sample.

2.5.3.1 Experiment 8. Pure Xylindein (CA11657 and CA7615) on separate 96-wells

Up to 10 ml of pigment-containing media was loaded onto an activated SPE column, so the target pigment could interact with the sorbent and form a visible green pigment band while the other metabolites were flushed out from the column by vacuum. When the column contents reduced to 3 ml, 3mL HPLC grade water was added to flush the undesired metabolites.

The contaminants were removed from the column by adding 10mL 50% acetonitrile in HPLC grade water. In the last step, 2 to 4mL of 100% chloroform or dichloromethane was added to remove the green pigment from the SPE sorbent. Then, the purified green pigments from *Chlorociboria aeruginosa,* and *Chlorociboria aeruginascens* were tested on 72 embryos (72 embryos/ pigment) (figure 2).

2.5.3.2 Experiment 9. Pure Red Pigment (11517)

Up to 10 ml of pigment-containing media was loaded onto an activated SPE column so the target pigment could interact with the sorbent and form a visible red pigment band. The other metabolites were flushed from the column by vacuum. When the pigment-containing media was close to less than one-third of the column, 3mL HPLC grade water was used to flush the contaminated metabolites from the column.

Contaminants were removed by adding 10mL 50% acetonitrile in HPLC grade water. Two to 4mL of 100% chloroform or dichloromethane solvent was added to separate the red pigment from the SPE sorbent. Then the purified red pigment was applied on 72 embryos to determine acute toxicity (figure 2).

2.6 Assessment

The assessment method modified from Troung et al. (2011) was used to observe the growth of the embryo at 24 hpf and to look for developmental stages and natural forms of kinetics (earliest behavior of zebrafish embryos). The timing of development was considered abnormal if the zebrafish embryos were more than 12 hours delayed compared to the control animals. Spontaneous forms of kinetics were assessed over two-minute periods and any lack of embryonic contractions or movement was noted. At 120 hpf, embryo morphology including body axis, ocular perceiver, snout, jaw, notochord, heart, brain, somite, fin, yolk sac, trunk, circulation, pigment, swim bladder, and behavioral endpoints (motility, tactile replication) were observed and recorded. Mortality rate was recorded at 24 and 120 hpf (Troung, 2011).

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Figure 1. The distribution of 100% concentration of each pigment extract and control in the 96-well plate.

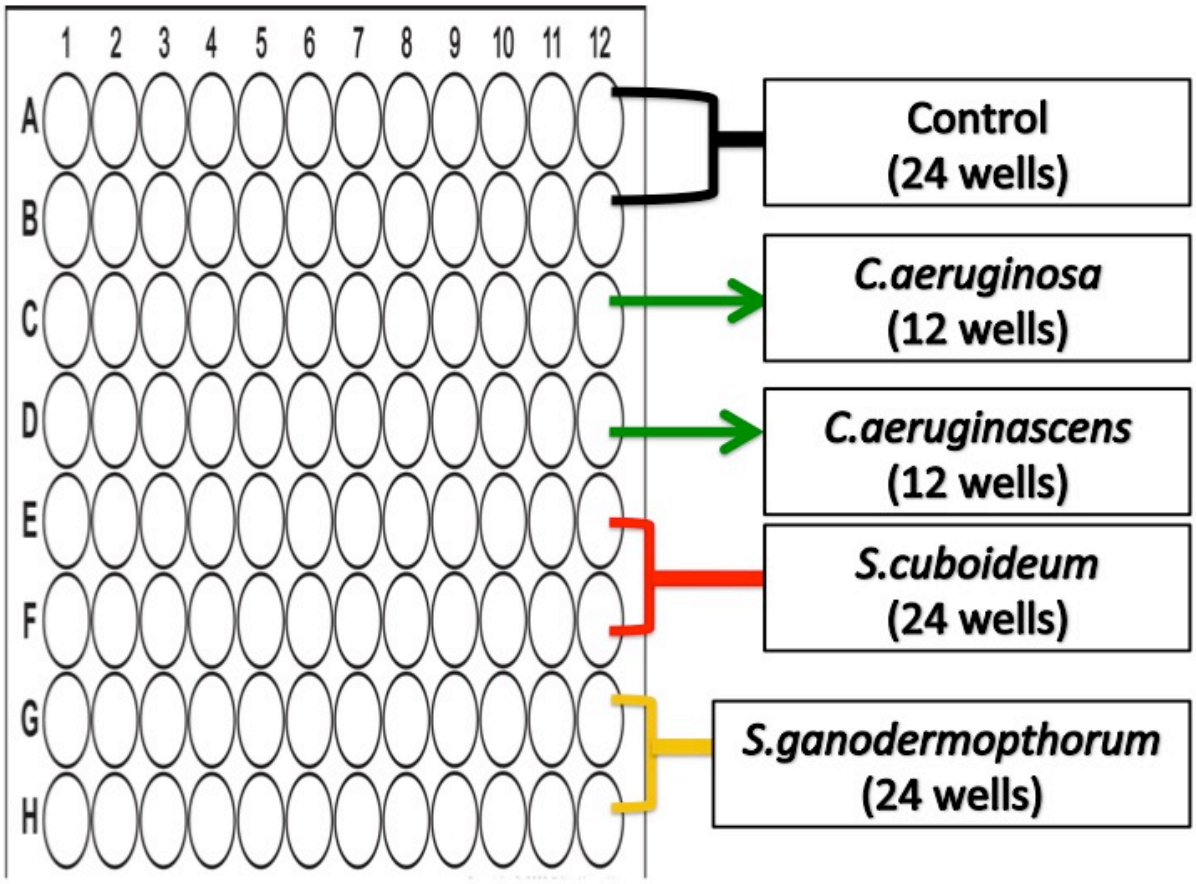


Figure 2. The distribution of 100% or 200% or 400% concentration or purified red or green pigment (72 embryos/pigment) and controls in each 96-well in Experiments 2, 3 and 4.

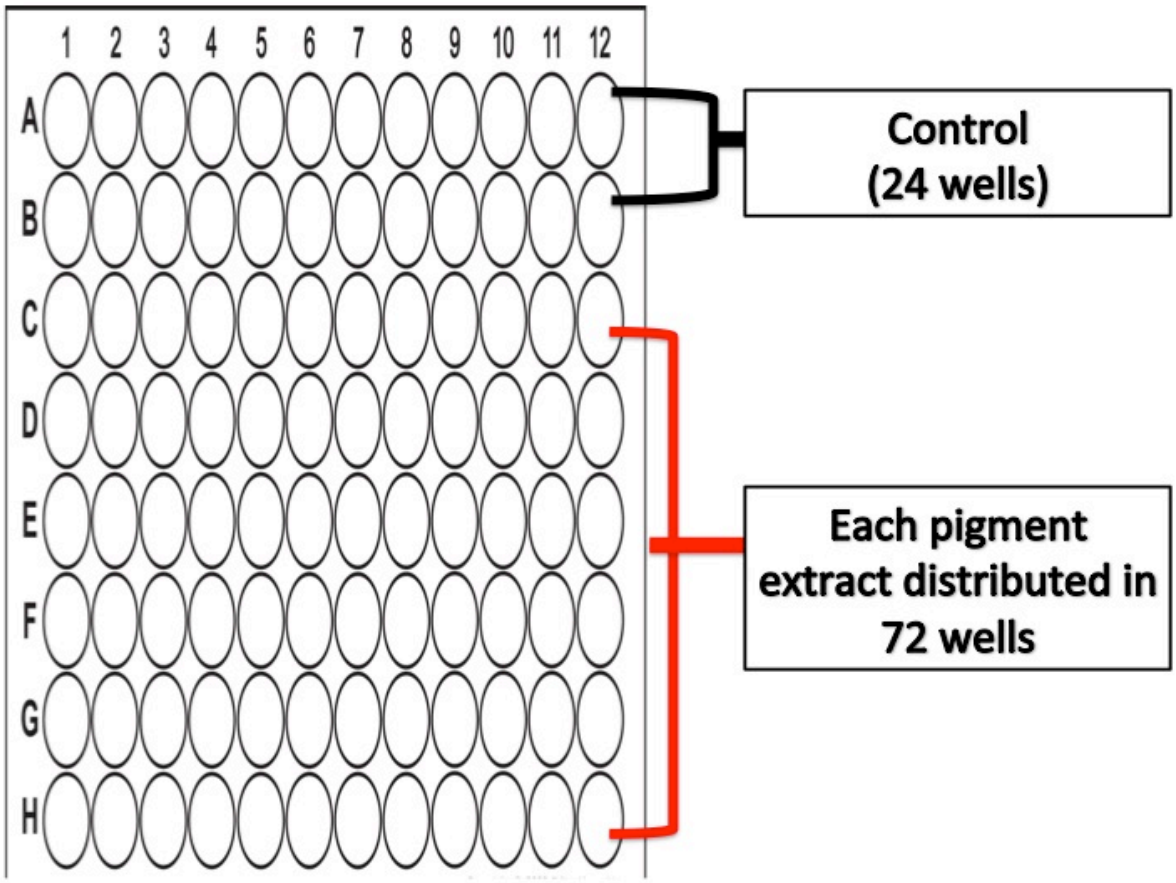


Figure 3. The distribution of each pigment in live or Autoclaved liquid malt and control in 96-well plate (12 embryos/ pigment) - the crossed G&H wells were not used.

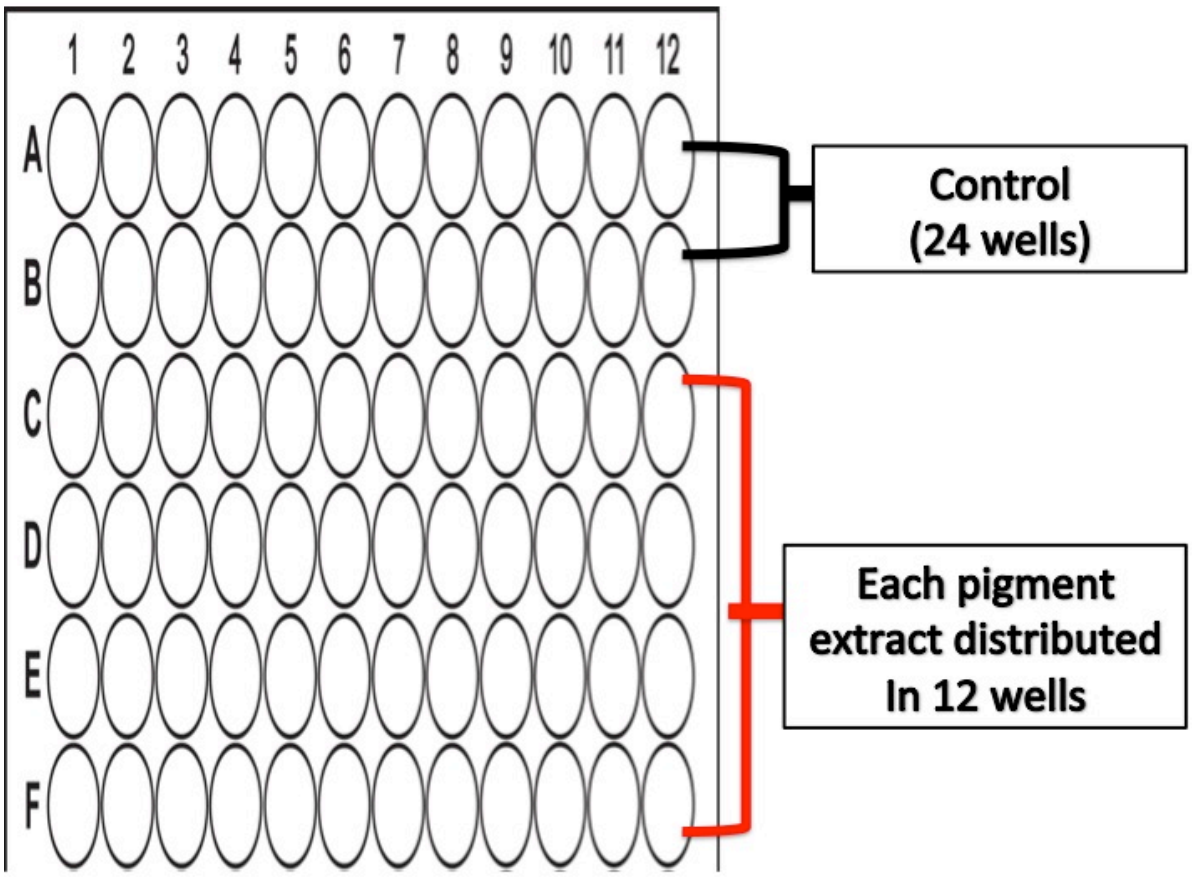
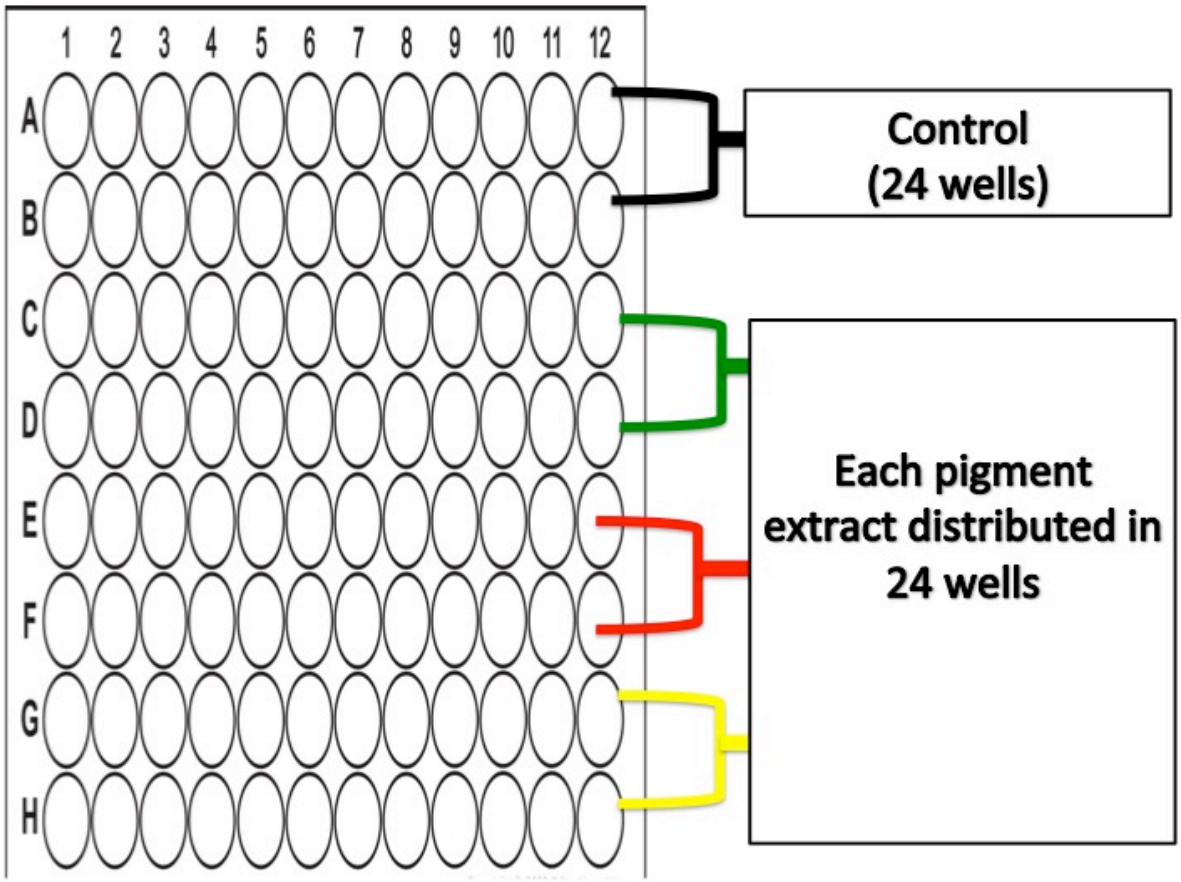


Figure 4. The distribution of each pigment filtered liquid malt and controls in 96-well plate (24 embryos/ pigment).



3.0 Results

Across all tests, there were no differences between the two controls after 24hpf and 120 hpf (5 days) and therefore the controls were combined together for statistical analysis.

3.1 Result 1: Wood Agar 100% Concentration. First Run (all pigment extract on the same 96-well PLATE):

There was no evidence that the zebrafish development on the extracted pigment solutions from any of the test fungi differed from that of the control after 24 hours (table 1). However, there was strong evidence that the toxicity of pigment extracts from the *Chlorociboria* species and *S. cuboideum* were different than the control after 5 days. Embryos exhibited significant deformation and mortality (Tables 1 & 9).

There was no evidence that the toxicity of the extracted yellow pigment from *Scytalidium ganodermophthorum* was different from the toxicity of the control after 5 days (Table 1).

3.2 Result 2: Wood Agar- 100 % Concentration, Second Run (all dyes on separate wells):

There was strong evidence that the toxicity of the pigment extracts from *Chlorociboria aeruginosa*, *Chlorociboria aeruginascens* and *Scytalidium cuboideum* differed from the recorded toxicity of the controls after 24 hours (Table 2). There was no evidence that toxicity of yellow pigment from *Scytalidium ganodermopthorum* was different than controls after 24 hours (Table 2).

After five days, there was strong evidence that the toxicity of all the pigment extracts except *S. ganodermophthorum* differed from the toxicity of the control. Embryos exhibited significant deformation and mortality (Tables 2 & 9). There was no evidence that the yellow pigment extract from *Scytalidium ganodermophthorum* differed from the control in terms of the observed toxicity after 5 days (Table 2).

3.3 Result 3: Wood Agar 200% Concentration (all dyes on same well, different numbers of controls and treatments than before):

There was substantial evidence that the toxicity of all the pigment extracts differed from the toxicity of the control after 24 hours (Table 3) and five days. Embryos exhibited significant deformation and mortality (Tables 3 & 9).

3.4 Result 4: Wood Agar – 400% Concentration (all dyes on same well, different numbers of controls and treatments than before): A summary of the results illustrated in Table 4 & 9

There was strong evidence that the recorded toxicity of all the pigment extracts differed from the toxicity of the control after 24 hours (Table 4) and five days. Embryos exhibited significant deformation and mortality (Tables 4 & 9).

3.5 Result 5: Liquid Malt Test (alive culture-all pigment solution examined on same 96-well): A summary of the results illustrated in Table 5

There was no evidence that the green pigment extracts from *Chlorociboria aeruginosa* and *Chlorociboria aeruginascens* differed from the control in terms of the observed toxicity after 24 hpf (Table 5).

There was strong evidence that the pigment solutions from *Scytalidium cuboideum* and *Scytalidium ganodermophthorum* differed from the controls in terms of the observed toxicity after 24 hours. For both pigment solutions, all of the embryos were dead (Table 5).

After five days, there was strong evidence that all pigment solutions differed from the control. Embryos exhibited significant deformation and mortality (Table 5).

3.6 Result 6: Autoclaved Liquid Malt (all dyes on same well): A summary of the results illustrated in Table 6.

There was no evidence that the green pigment solution from *Chlorociboria aeruginosa* differed from the control in terms of toxicity after 24 hours and all of the embryos were normal (Table

6). There was strong evidence that the toxicity of pigment solutions from *Chlorociboria aeruginascens*, *Scytalidium cuboideum* and *Scytalidium ganodermophthorum* differed from the control after 24 hours (Table 6). After five days, all embryos were dead for all pigments solutions (Table 6).

3.7 Result 7: Liquid Malt 0.2 Micron Filtered (duplicate samples runs):

There was no evidence that the toxicity of pigment solutions from *Chlorociboria aeruginosa* and *Scytalidium ganodermophthorum* were significantly different from the control after 24 hours. All of the embryos were normal (Table 7). There was strong evidence that the pigment solutions from *Chlorociboria aeruginascens* and *Scytalidium cuboideum* differed from control after 24 hours (table 7). After five days, there was strong evidence that the toxicity of the all pigment solutions differed from the control. All embryos were dead and deformed after exposure to the extracts (Table 7).

3.8 Result 8: Pure Xylindein from CA11657 CA7615 on separate 96-wells: A summary of the results illustrated in Table 8

There was strong evidence that the toxicity of green pigment from *Chlorociboria aeruginosa* and *Chlorociboria aeruginascens* were significantly differed from the control after 24 hours and five days (Table 8). Embryos exhibited significant deformation and mortality (Table 8).

3.9 Result 9: Pure Concentration Red Pigment (For *Scytalidium cuboideum*)

There was no evidence that red pigment differed from the controls in terms of toxicity after 24 hours and all embryos were normal (Table 8).

After five days, there was strong evidence that the red pigment from *Scytalidium cuboideum* differed from the control. Embryos exhibited significant deformation and mortality (Table 8).

Table 1. Effects of the fungal pigment extracts on development and mortality of zebrafish embryos - 100% pigment concentration (aspen wood medium).

Exposure time	*C. aeruginosa* (UAMH 11657)			*C. aeruginascens* (UAMH 7615)			*S. cuboideum* (UAMH 11517)			*S. ganodermophthorum* (UAMH 10320)		
	Death	Delay progression	Deformed	Death	Delay Progression	Deformed	Death	Delay Progression	Deformed	Death	Delay Progression	Deformed
24 hpf	0%	0%	-	0%	0%	-	0%	0%	-	0%	0%	-
120 hpf	75%	-	100%	100%	-	100%	25%	-	46%	0%	-	0%

Table 2. Effects of the fungal pigment extracts on development and mortality of zebrafish embryos - 100% pigment concentration (Maple wood medium).

Exposure time	*C. aeruginosa* (UAMH 11657)			*C. aeruginascens* (UAMH 7615)			*S. cuboideum* (UAMH 11517)			*S. ganodermophthorum* (UAMH 10320)		
	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed
24 hpf	49%	36%	-	43%	57%	-	100%	0%	-	0%	0%	-
120 hpf	78%	-	98%	100%	-	100%	100%	-	-	0%	-	0%

Table 3. Effects of the fungal pigment extracts on development and mortality of zebrafish embryos - 200% concentrate from 100% (Maple wood medium).

Exposure time	*C. aeruginosa* (UAMH 11657)			*C. aeruginascens* (UAMH 7615)			*S. cuboideum* (UAMH 11517)			*S. ganodermophthorum* (UAMH 10320)		
	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed
24 hpf	44%	56%	-	0%	100%	-	7%	25%	-	66%	17%	-
120 hpf	100%	-	100%	100%	-	100%	28%	-	92%	83%	-	83%

Table 4. Effects of the fungal pigment extracts on development and mortality of zebrafish embryos - 400% concentrate from 200% (Maple wood medium).

Exposure time	*C. aeruginosa* (UAMH 11657)			*C. aeruginascens* (UAMH 7615)			*S. cuboideum* (UAMH 11517)			*S. ganodermophthorum* (UAMH 10320)		
	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed
24 hpf	50%	50%	-	41%	58%	-	33%	66%	-	92%	0%	-
120 hpf	100%	-	100%	100%	-	100%	100%	-	100%	100%	-	100%

Table 5. Effect of live medium from culture of selected pigments produced by fungi on zebrafish development and mortality after 24 and 120 hpf.

Exposure time	*C. aeruginosa* (UAMH 11657)			*C. aeruginascens* (UAMH 7615)			*S. cuboideum* (UAMH 11517)			*S. ganodermophthorum* (UAMH 10320)		
	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed
24 hpf	0%	0%	-	0%	0%	-	100%	0%	-	100%	0%	-
120 hpf	50%	-	0%	100%	-	100%	100%	-	100%	100%	-	100%

Table 6. Effect of autoclaved medium from culture of selected pigments produced by fungi on zebrafish development and mortality after 24 and 120 hpf.

Exposure time	*C. aeruginosa* (UAMH 11657)			*C. aeruginascens* (UAMH 7615)			*S. cuboideum* (UAMH 11517)			*S. ganodermophthorum* (UAMH 10320)		
	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed
24 hpf	0%	0%	-	0%	100%	-	100%	0%	-	100%	0%	-
120 hpf	100%	-	100%	100%	-	100%	100%	-	0%	100%	-	0%

Table 7. Effect of filtered medium from culture of selected pigments produced by fungi on zebrafish development and mortality after 24 and 120 hpf.

Exposure time	*C. aeruginosa* (UAMH 11657)			*C. aeruginascens* (UAMH 7615)			*S. cuboideum* (UAMH 11517)			*S. ganodermophthorum* (UAMH 10320)		
	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed
24 hpf	0%	0%	-	29%	71%	-	100%	0%	-	0%	0%	-
120 hpf	100%	-	100%	100%	-	100%	100%	-	0%	100%	-	100%

Table 8. Effect of purified extracts from *C.aeruginosa, C.aeruginascens and S.cuboideum* pigments on zebrafish development and mortality after 24 and 120 hpf.

Exposure time	*C. aeruginosa* (UAMH 11657)			*C. aeruginascens* (UAMH 7615)			*S. cuboideum* (UAMH 11517)		
	Death	Delay progression	Deformed	Death	Delay progression	Deformed	Death	Delay progression	Deformed
24 hpf	0%	100%	-	0%	100%	-	0%	0%	-
120 hpf	63%	-	97%	71%	-	100%	70%	-	85%

Table 9. Abnormal morphology of zebrafish embryos for each Agar wood-DCM pigment extract by species after 120 hpf.

Concentrations	100% pigment concentration (Aspen wood medium)	100% pigment concentration (Maple wood medium)	200% concentrate from 100% (Maple wood medium)	400% concentrate from 200% (Maple wood medium)
Species	Observed abnormal morphology			
Chlorociboria aeruginosa	Stunting, pericardial edema, yolk sac edema and axis reduced total length	Pericardial edema, yolk sac edema, and axis problem reduced total length	Pericardial edema, yolk sac edema, and axis problem	Yolk sac edema, reduced body length pericardial edema, deflated swim bladder, bent tail, and bent trunk.
Chlorociboria aeruginascens	Stunting, pericardial edema, yolk sac edema and axis reduced total length	Pericardial edema, yolk sac edema, and axis problem reduced total length	Pericardial edema, yolk sac edema, and axis problem	Yolk sac edema, reduced body length pericardial edema, deflated swim bladder, bent tail, and bent trunk.
Scytalidium cuboideum	Pericardial edema, yolk sac edema and axis defect	Dead before achieving 120 hpf stage	Pericardial edema, yolk sac edema, reduced total length	Pericardial edema, yolk sac edema, trunk, axis and head problems
Scytalidium ganodermophthorum	-	-	Pericardial edema, yolk sac edema, reduced total length	Pericardial edema, yolk sac edema, trunk, axis and head problems

4.0 Discussion

Zebrafish embryo acute toxicity tests were used in this study as an *in vivo* model to screen the adverse effects of fungal pigment mixtures because of a future commercial plan to expose the pigment mixture to humans and the ecosystem. Purified forms of the pigments are still under development and as such the main aim of this study was to assess the safety of the pigment mixtures.

Results differed between toxicity thresholds from the DCM-extracted pigments and the liquid culture pigments. There are several potential explanations for these differences, as outlined below.

The DCM pigment extracts exhibited toxic effects at concentrations of 100%, 200%, and 400% due to possible interactivities between the ingredients of the mixtures. When chemicals interact in a mixture, they can alter the toxic effects of one another and can be categorized under classes of toxic reactions: additive, synergetic (Berenbaum, 1989), antagonistic (Boik, 2008) or potentiating (COT, 2002). These types of interactivity may consequently vary depending on relative concentrations, the routes of exposure, the time and duration of exposure including the biological persistence of the mixture components, and the biological targets. This phenomenon is considered common in environmental mixtures (Gregoraszczuk, 2008).

The DCM pigment extracts of *Chlorociboria aeruginosa*, *Chlorociboria aeruginascens* and *Scytalidium ganodermophthorum* (figure 5) showed an additive type of interactivity. The additive effect is the sum of the effects of the chemical involved in the reaction. This effect is common between chemicals of similar structure, which may work together (Berenbaum, 1989; Boik, 2008; COT, 2002). In the additive effect, the increase of the pigment extracts concentration increases the toxic effects. The study results indicated significant abnormality and mortality after 24 and 120 hpf in embryos in response to higher concentrations of the pigment mixtures.

The DCM pigment extract of *Scytalidium cuboideum* exhibited a different kind of interactivity. This pigment extract showed antagonistic interactivity at higher concentrations (figure

6). The antagonistic effect is when the net effects are equal to zero, which leads to a neutralization of the effect and causes no harm or reduces the harm (Berenbaum, 1989; Boik, 2008; COT, 2002). The reduction of deformations and mortality percentage in the higher concentrations of the pigment mixture may indicate the antagonistic effect. At the higher concentrations, the chemical ingredients of the red pigment mixture might modulate and repress the effects of each other. Some of the ingredients might compete on the same active sites in the cells of the embryos. This can lead to gene expression and different cell responses. In this case, the gene expression and responses may have reversed the toxic response of potent ingredients of the mixture (Liebler,1993; Freedman,1995).

Mortality rates were higher after zebrafish were exposed to live liquid cultures than in the DCM cultures. This could be due to the toxic effects caused by ingredients in the medium, the pigment, other metabolites or caused by the live cultures themselves. The toxicity may be caused by production of different enzymes and secondary metabolites from these fungi due to stress induced by the fish water medium. The sudden changes in environmental conditions can force fungi to produce different toxic secondary metabolites to adapt to the new conditions (Zeilinger, 2015).

Autoclaved pigments from live cultures proved to be the most toxic (100% mortality). It is possible that the heat from the autoclave broke down the parent chemical contents of the pigment solutions to produce more toxic compounds (Irwin, 1997). This degradation should be considered as an environmental factor that might be replicated in the ecosystem.

For the filtered cultures, although the filtration techniques may have reduced the quantities of chemical constituent, toxic effects were still exhibited after 24 hpf and/or 120 hpf. The filtered medium may have contained toxic ingredients, including the pigment, from the beginning. Alternatively the fungal hyphae were disrupted during the vacuum filtration technique and released different chemical ingredients that passed through the filtrate and induced toxic effects to the exposed embryos. The biosynthesis mechanism of different metabolites is known as ‘sensitive to environmental signals’, including stressors such as pressure, temperature, light and pH (Berry, 1988).

Overall, it is difficult to make assumptions about the toxicity differences in the DCM pigment extracts versus the liquid malt. There were a number of additional variables in each extract that could have been responsible for the differing toxicity, such as wood extractives from the wood media base in the DCM extracts, and the live fungal hyphae in the liquid culture. Wood extractives in the DCM pigments originated from the wood shavings used in the nutrient growth media (aspen (*Populus*) and maple (*Acer* spp.)). In contrast, no wood was used in the liquid culture media. Growth medium can play a huge role in the production of secondary metabolites (Henkenn, 2003). The media can induce or regulate signals that activate a "master gene" in fungal species, which controls the production and differentiation of secondary metabolites (Demain, 1998). It is likely that the DCM pigment extracts contained different compounds than the liquid cultures, in terms of wood extractives and the resulting fungal secondary metabolites. This, in turn, may have led to the differences in toxicity between the two pigment types.

Another explanation for the difference in toxicity between the liquid and solid cultures is that the liquid malt pigments may contain ingredients that are more bioavailable to the exposed embryos than the DCM- pigment due to higher water solubility. Chemical existence in environmental samples is not always an indication of bioavailability (Huckins, 1990; Hamelink, 1994). The solubility of the mixture contents can control bioavailability. If the chemical is bioavailable, it can be taken by the exposed organism and cause biological effects (Connell, 1990). As the pigments in the liquid culture were suspended in water, versus the DCM pigments that were suspended in DCM and then deposited on the glass plate, it is likely that the water-suspended pigments were more bioavailable. These fungal pigments are known to be hydrophobic and bind readily to their glass substrate, making the bound components less bioavailable. The seemingly 'lower' toxicity from the DCM pigments may be due to the extra time it took the pigments to resolubilize into the fish water and become bioavailable (and therefore toxic).

Although artisans have used spalted wood for hundreds of years, there is no recorded instance of toxicity to woodworkers. This would seem to be in contradiction to these findings. An

interpretation to this contradiction is that the influence of these pigments might be dissimilar to humans because the sensitivity level might be different between humans and zebrafish embryos. Furthermore, the route of exposure in human to the pigments might differ than the rout of exposure in zebrafish embryos.

It is possible that pigment extracts in this study may have produced secondary metabolites different from their wild counterparts due to their growth conditions. Fungal growth in the laboratory is usually done via asexual reproduction and sporulation, which can lead to different secondary metabolites than those that are produced in sexual reproduction (Luckner et al., 1977). Manipulating of fungal growth rate can regulate productions of different secondary metabolites as well (Shuler, 1981). The improvement and optimization of culture conditions in laboratories can induce fungal species to alter secondary metabolite production due to gene expression and clustered genes and may produce different secondary metabolites (Righelato, 1967). All growth processes used in the lab to generate pigment were optimized for fast growth, and caused the fungi to grow and pigment much more quickly than they would have in the wild. It is possible that these stressful growing conditions increased the amount and variation of secondary metabolites produced. Secondary metabolites produced under these unusual stress conditions might include more potent toxins than would be produced under normal growth conditions

Overall, all the pigments exhibited some level of toxicity over time. Whether this was due to the pigments themselves, or some other component of the mixtures is undetermined. Future research in this area should focus on isolation and purification techniques so that toxicity tests can be run on only the isolated pigments to definitively determine toxicity of these pigments to living organisms, and their suitability as colorants.

Adopting the use of natural fungal pigments is a major challenge. In addition, companies have to face the challenge of whether their products will be readily accepted by consumers. Additional testing in this area could also focus on comparative toxicology between these fungal pigments and

their aniline dye counterparts. If the fungal pigments are no more toxic than their synthetic counterparts, consumers may be more willing to accept the products.

One of major issues with synthetic dyes is color effluent from the dyeing process and contamination of the ground and surface water in the environment (O'Neill, 1999; Wang, 2007). Public concerns are also increasing about synthetic dyes as causative agents for health problems. Some synthetic dyes have recently been dropped from the market because they were found to be a source of disease, hazardous waste, and involved in producing green house gases (Francalanci, 2001). Therefore, it is important to find better economically and environmentally friendly alternatives, such as fungal pigments. The replacement of synthetic dyes with natural pigments is a promising approach to reduce pollution (Duran *et al.*, 2002), as many demonstrate good biodegradability and higher compatibility with the environment (Gupta *et al*., 2001; Duran *et al.*, 2002).

Continued research on the safety of fungal pigments will benefit major industries and create numerous opportunities for employment. This research was the first step towards understanding how these pigments affect animals which, in turn, will dictate how and when they can be used. Additional research in this area could help bring these pigments into commercial development and eventually modernize the dye industry, should their toxicity become better understood.

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Figure 5. Example of mixture additive effect- comparison between 100%, 200% & 400% concentrations of yellow pigment extracts from Maple wood (*Acer saccharum*) medium on mortality effect in exposed zebrafish embryos after 24 & 120 hpf.- Error bars represent standard error.

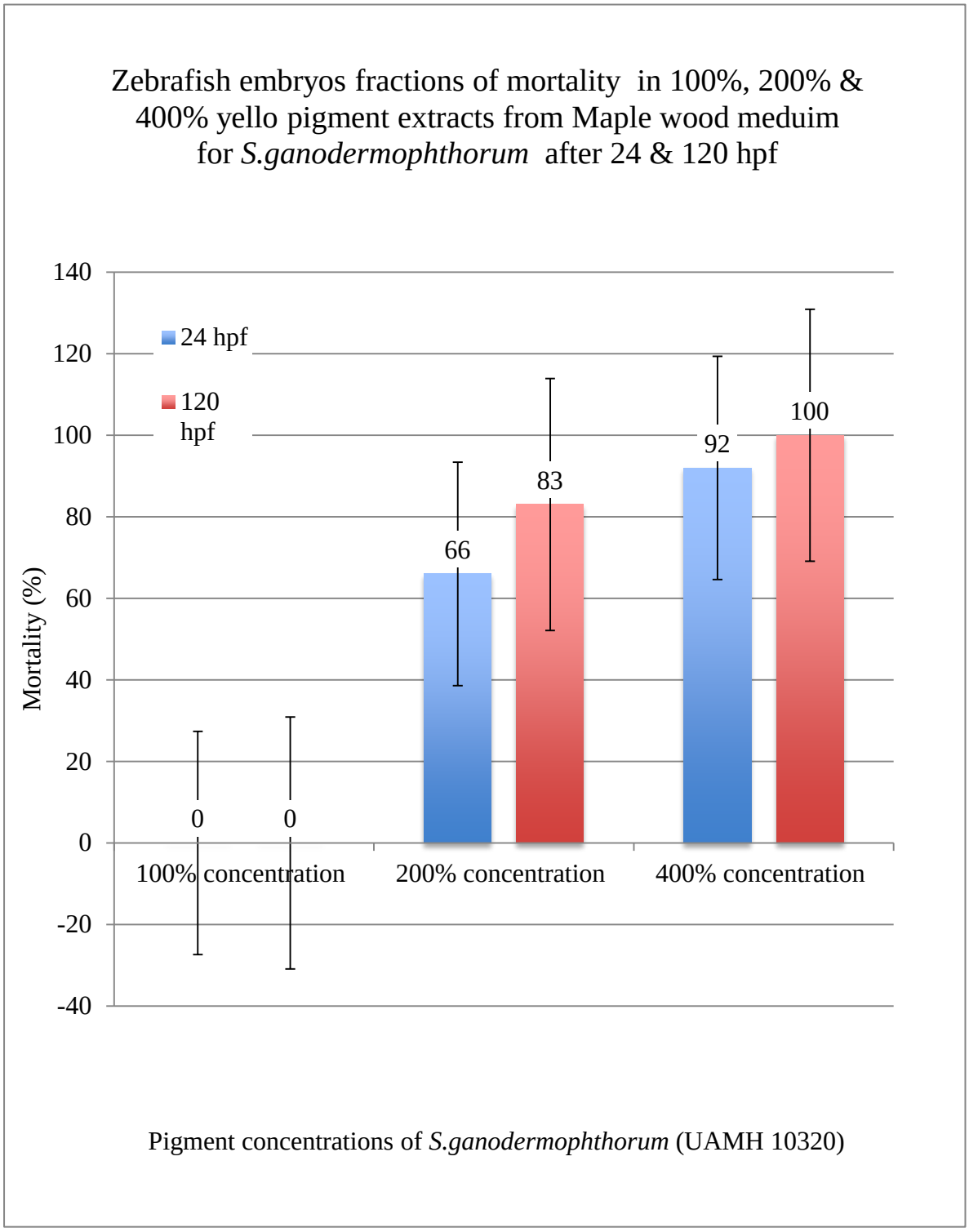
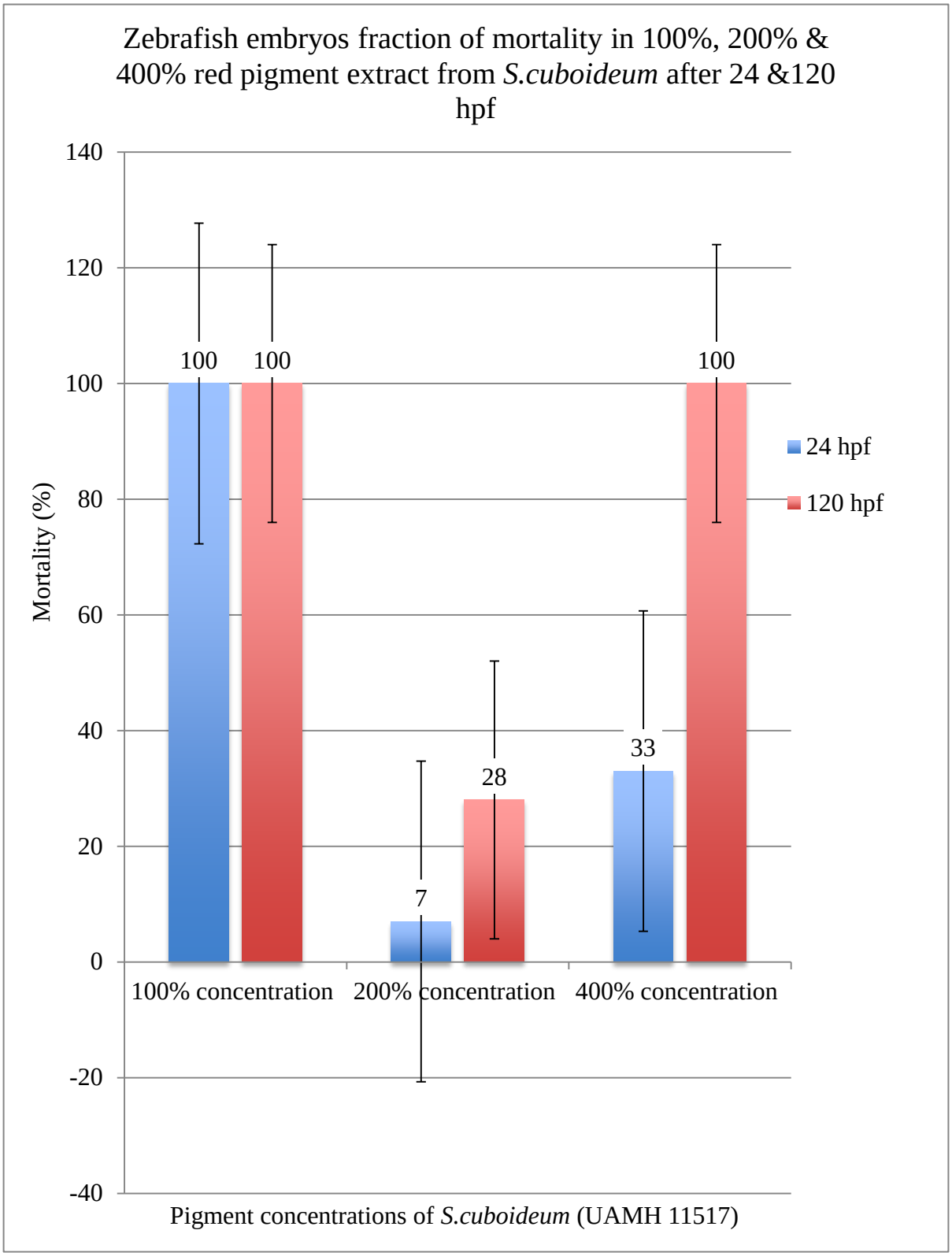


Figure 6. Example of antagonistic effect- comparison between 100%, 200% & 400% concentrations of red pigment extracts from Maple wood (*Acer saccharum)* medium on mortality effect in exposed zebrafish embryos after 24 & 120 hpf.- Error bars represent standard error.



5. Conclusion

This research studied abnormalities in zebrafish following exposure to pigment mixtures from solid culture DCM extracts, liquid malt pigments and purified pigments from fungi. It was difficult to provide data on the action of individual compounds, the nature of the action, or interaction between individual compounds since a mixture was used, even in the 'purified' compounds. However, the study results did provided practical insight on fungal pigment mixture toxicity and its effect on living organisms. Based on the results of this research study, the DCM pigment extracts and liquid malt pigments exhibited variable toxicity because of variations in the types of interactivity. These types of interactivity depend on many factors such as different concentration levels, the routes of exposure, and the time and duration of exposure.

Furthermore, because of the high uncertainty factor about the ingredients within each mixture, it was difficult to understand the relationship between the toxic effects and each individual pigment in the mixture. It was hard to determine if DCM pigment extracts could be more toxic or less or equal to liquid malt pigment toxicity. Both types of pigment mixtures caused high mortality rates and deformation. Despite the presence of the pigments in higher concentration in liquid malt mixtures than the DCM pigment extracts, it was hard to conclude that the toxicity was due to the individual pigment. The presence of wood extractives in DCM pigment extracts and other unknown secondary metabolites in both DCM pigment extracts and liquid malt pigments made it impossible to confirm the toxicity of individual pigment. Future work should focus on purifying the targeted pigments individually to confirm the relationship between each pigment and any adverse effects. Furthermore, the purification should be followed by determination of the toxic dose response, if present. Future studies to understand the relationship between the pigments and toxic effects could play an important role in making decisions about the potential use and handling of fungal pigments from these *Chlorociboria* and *Scytalidium* species.

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