Study on diets for Japanese medaka fish to reduce cadmium toxicity

(カドミウム毒性を低減するためのメダカの食餌に関する研究)

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May, 2021

List of abbreviations

AAS	Atomic Absorption Spectroscopy
ANOVA	Analysis of variance
ATSDR	Agency for Toxic Substances and Disease Registry
CAT	Catalase
Cd	Cadmium
DCT	Divalent Cation Transporter
DMT	Divalent Metal Transporter
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
GD	Garlic diet
GSH	Glutathione
IAA	In diet antioxidant ability
K	Condition factor
LPO	Lipid peroxidation
MD	Medaka diet
MDA	Malondialdehyde
MDG	Millennium Development Goal
MRP	Multidrug Resistant Protein
MT	Metallothionein
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PD	Propolis diet
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDG	Sustainable Development Goal
SOD	Superoxide dismutase
WD	Wakame diet

Acknowledgements

I owe all thanks to God Almighty, the author and finisher of my faith. His never ending love, compassion and grace have seen me through this doctoral program. I must thank my supervisors, Professors Masaaki Kurasaki and Tatsufumi Okino very deeply. Their tutelage, forbearance, guidance and mentorship were a necessary force that saw me through the conclusion of my studies. 本当に感謝しています! Sincere thanks are also due to the professors in Environmental Adaptation Science. Their guidance, dedication, presence, especially during seminars and presentations, questions and comments, led me through the right paths, and formed a strong core towards the successful completion of this program.

All lab mates from Kurasaki and Okino laboratories, past and present are thanked, individually and collectively for every role they played. The network we shared during the course of study, and encouragements in all forms they came are sincerely appreciated. Special thanks are due to Drs. Tajuddin Sikder, Mostafiz Rahman, Subrata Banik and Rachael Lopez for their time and solicited explanations and criticisms. They were kind to me. Ms Serene Bondad was a close buddy and is thanked immensely for sacrificing her time during those demanding laboratory analysis, explanations and criticisms. I relish her gifts of special home-made cookies. I pray God's blessings on them all.

My lovely wife Godsfavour, and son Justin are deeply appreciated and thanked. They tolerated my incessant absence and late nights, necessary towards the completion of my laboratory analysis and write-up of this dissertation. They complete my world, and giving up is never an option. To my parents Elder (Chief) Okechukwu Ujeh and Mrs. Chinyere Ujeh, my siblings Ahiaoma, Ngozi, Chinenye, and Kelechi, and their families, and my parents' in-law, I say a huge thank you. Your intercessory prayers on my behalf and love have always been my strength. I love you all. All members of Seventh Day Adventist Church, Sapporo are deeply thanked for their love, fellowship, and prayers. Members of Hokkaido University African Student's Association formed a great backbone of brotherhood in a foreign land and are deeply thanked.

The erstwhile vice chancellor of Ebonyi State University, Prof Francis Idike, and the university senate are thanked for approving my selection as the university's candidate for Hokkaido University President's Fellowship, 2016. Prof. Dr. Johnny Ogunji, my MSc supervisor is particularly thanked for his indelible mark in my student life. He taught me the rudiments of writing scientific reports, research papers, and statistical analysis. He mentored me.

Lastly, this study, and my sojourn in Japan was possible because of the Hokkaido University President's Fellowship, 2016 which I received. It unlocked endless possibilities for me and marked me for greater achievements and fulfillments. It was a dream come true and my life has been affected positively. I am eternally grateful for the award!

Abstract

Emerging evidences show increasing trends of aquatic environmental pollution which undermine pristine water conditions and the support to fish and aquatic lives. Aquatic environments are not only pickers of anthropogenic contaminants, but also serve as their final destination. Cadmium (Cd) is listed as a priority heavy metal pollutant of aquatic environments. It is released into the aquatic environment through numerous natural and anthropogenic activities. It is non-biodegradable, persistent, cumulative, and toxic to biological tissues. Fishes are designated as 'sentinel species' of aquatic environmental pollution due to their high vulnerability to Cd and other aquatic contaminants. Various water treatment methods usually accompany water pollution cases, but little or no attention is given to surviving fish thereafter. Moreover, Cd induced effects can be protracted, defying the protection by endogenous antioxidants and defense mechanism in fish. Such scenarios potentially lead to impaired productivity and decline in fish population, Cd biomagnification along successive links in aquatic food chains, and increased Cd exposure risk on fish consumers. This necessitates the need to enhance recovery from tissue accumulation and Cd toxicity effects in surviving fish, following Cd exposure. The context of this study identifies two major categories of effects on exposed fish, viz i) accumulation effects; and ii) toxicity effects. These categories constitute chapters 2 and 3, respectively in this dissertation. In fish, various plants and plant derived compounds have been widely researched for their growth promoting and antioxidative effects. Their contribution however, to recovery from metal exposure and toxicity effects is yet to be fully addressed. Based on their immune stimulating and antioxidative merits, garlic, propolis, and wakame were selected for this study. Their ability to reduce toxicity and promote general recovery in fish previously exposed to Cd was assessed. The experimental model, Japanese medaka (*Oryzias latipes*) was used for this study based on its numerous research qualities – it is small, cost-effective, and easy to breed in large numbers, prolific, and can be confined easily. Most experimental tools for the analysis of gene function can also be applied to the species. However, its welfare and recovery dynamics from Cd and other environmentally relevant heavy metals is yet to be addressed. More so, as pollution by heavy metals is

almost inescapable to fish, it becomes crucial to examine its recuperation dynamics. Therefore, the main purpose of this study was to assess the effects of garlic, propolis, and wakame supplemented diets on recuperation in Cd exposed Japanese medaka fish. Two broad aims of the study were i) to assess ability of the experimental diets to improve Cd depuration in tissues of exposed Japanese medaka fish; ii) to assess ability of the experimental diets to ameliorate Cd induced toxicity within tissues of exposed Japanese medaka fish. To achieve these aims, two set of investigations were carried out as chapters two and three, respectively.

The specific objectives of chapter two were to comparatively assess i) Cd bioconcentration, and ii) Cd depuration in exposed medaka fish juveniles under three dietary regimes. Fish were exposed to 0.3 mg Cd /L in water for 21 days under a control diet. Surviving fish were further depurated for 21 days and fed garlic (GD at 4%), propolis (PD at 4%), wakame (WD at 5%) supplemented diets, or medaka diet (MD at 100 %) as control diet. Fish gills, liver, and muscles were assessed weekly for Cd bioconcentration and depuration by atomic absorption spectroscopy (AAS); and metallothionein (MT) induction by enzyme linked immunosorbent assay (ELISA). Dietary performance results showed fish biometrics and condition indices in GD, PD, and WD compared favorably with those of MD. Following Cd exposure, Cd concentration and MT induction increased significantly across all sampled tissues. Conversely, treatments with GD, PD, and WD significantly reduced Cd concentration and upregulated MT expression across all tissues. Therefore, dietary supplementation supported Cd depuration and potentially reduced bioconcentration margin and associated health risks in the order garlic diet > wakame diet ≥ propolis diet > medaka diet. The specific objectives of chapter three were to comparatively assess i) Cd induced toxicity and ii) Cd toxicity amelioration under three dietary regimes. Experimental protocols were similar with those of chapter 2. Gill, liver, and muscle tissues were assessed weekly for the antioxidants superoxide dismutase (SOD) and total glutathione (GSH), and also lipid peroxidation (LPO) for toxicity effects. In addition, magnitude of recovery in fish was assessed through percentage mortality and survival, fish biometrics and condition factor (K). Results showed reduced antioxidant activity by significantly increasing LPO and

reducing SOD activity and GSH contents in gill and muscle tissues upon Cd exposure. In contrast, GD, PD, and WD diets significantly reduced LPO, while significantly increasing GSH, SOD activity, and K, in addition to reducing mortality and improving survival. Dietary supplementation therefore significantly increased recuperation and tissue functions in fish, in the order garlic diet > wakame diet ≥ propolis diet > medaka diet. In summary, dietary supplementation of garlic, propolis, and wakame are relevant in improving fish health status and recovery following incidences of Cd pollution and water treatment, especially in aquaculture settings. They are relatively cheap, affordable, and produced all year round, and are strongly recommended to fish farmers. Further studies to establish the molecular mechanism of Cd toxicity amelioration with garlic, propolis, and wakame *in vitro* are suggested.

Chapter 1: General Introduction

1.1.Background

Health is an invaluable asset to all living things. It is necessary for the proper functioning of the body, and attainment of life goals. A close synergy exists between the health of an inhabitant and its environment. The World Health Organization in 2016 estimated that over 20% of the global disease burden and deaths were attributed to modifiable environmental factors (Joubert *et al*., 2020). Today, environmental health issues occupy a place of prominence in the global political space. For example, in the Millennium Development Goals (MDG Goal 7), environmental sustainability formed one of the core goals, while in the Sustainable Development Goals (SDGs), it is spread between Goal 6 (Clean water and sanitation) through Goal 14 (Life below water). The environment then, is an invaluable asset, whose health not only influences those of its inhabitants, but which quality must also be courted very jealously!

The age long saying – "water is life", captures the all-important role of water in the sustenance of life in all its forms. The waters of the aquatic environment constitute about 71 % of the earth's surface. On average, the human body consists of about 60% water (Benelam and Wyness, 2010). The body of fishes on the other hand, contains approximately 70 – 84 % water (Abraha *et al*., 2018). Animals and fishes are not only

constituted of water in a large proportion, but also depend on it for survival. This all important role of water in supporting and sustaining life underscores the need to preserve its quality in aquatic environments.

Emerging evidences show increasing trends in aquatic environmental pollution which undermine pristine water conditions and the support to fish and aquatic lives (Hampel *et al*. 2015; Bhat *et al*. 2017). Aquatic environments are pickers for anthropogenic contamination and industrial wastes and leaks, whether chemicals or solid pollutants (Hampel *et al*. 2015; Bhat *et al*. 2017). Thus, they appear as the final destination for most anthropogenic contaminants released from industry, agriculture, urbanization, transport, tourism, and everyday life (Amiard-Triquet, 2015). Accordingly, and unfortunately so, aquatic organisms must confront all manner of waterborne wastes and pollutants. These contaminants cause various degrees of harm on aquatic inhabitants and generate health and safety issues due to their nature. Fish, for example carry out all biological and physiological activities in water as their immediate environment. They are therefore almost inescapable to aquatic pollution. Fish exhibit very high sensitivity to aquatic pollutants that they are considered sentinels of aquatic pollution (Pottinger *et al*., 2002; Christophe *et al*., 2015). Numerous experimental models including transgenic Zebrafish (Carvan *et al*., 2000) and medaka fish (Padilla *et al*., 2009) have been extensively employed in environmental toxicity studies. Polluted aquatic systems are then treated by first identifying and halting the source of contamination, thereby preventing further entry of toxicants. This is usually followed by varying water treatment methods. However, beyond use as sentinel species of aquatic pollution, deserving attention is seldom paid on surviving fish species, post water

pollution remediation. Thus, fish with incurred health challenges due to contamination are left unattended. Depending on the nature of contamination and pollutant, affected fish constitute a health and safety risk to fish consumers along successive links in the food chain, including man. The way and manner fish confronts and challenges aquatic environmental perturbations through water pollution determines their success in recuperation, and deserves attention. Recuperation is a term used to refer to the process of recovery from illness. Among aquatic pollutants, heavy metals raise important health and safety issues, and consequently, the need for recuperation or recovery from their effects.

1.2. Heavy metals and health and safety issues

Heavy metals are serious pollutants due to their toxicity, persistence in natural conditions and ability to be incorporated into food chains (Szefer *et al*., 1997; Klavins *et al*., 2000; Armitage *et al*., 2007; Sakan *et al*., 2009). Of the 92 elements which occur naturally on earth, about 30 of them (metal-(loids)) are considered as potentially toxic to humans and animals (Morais *et al*., 2012). The contamination chain of heavy metals almost always follows a cyclic order: industry, atmosphere, soil, water, foods and human (Morais *et al*., 2012). Reported sources of heavy metals in the environment include geogenic, industrial, agricultural, pharmaceutical, domestic effluents, and atmospheric sources (He *et al*., 2005). Because of their high degree of toxicity, arsenic, cadmium, chromium, lead, and mercury rank among the priority metals that are of public health significance (Tchounwou *et al*., 2012). According to the ranking by United States Agency for Toxic Substances and Disease Registry (ATSDR, 1999), cadmium ranks as the seventh most widely

distributed environmental pollutant from natural or industrial sources (Sabeen *et al*., 2013; Mead, 2010).

1.3. Background on cadmium

Cadmium (Cd) is considered a major chemical pollutant of the aqueous environment and a serious threat to water organisms, especially fish (Directive, 2013). Environmental exposure to Cd may lead to the absorption of large quantities of the element, causing harm to the organism (Abalaka 2015). Cd occurs in the earth's crust at a concentration of 0.1–0.5 ppm and is commonly associated with zinc, lead, and copper ores (Faroon *et al*., 2012). Important anthropogenic sources of Cd include mining, atmospheric deposition of combustion emissions, and the use of Cd-containing fertilizers (Kubier *et al*., 2019). In soil water however, Cd usually occurs up to 5 μg/L (Smolders and Mertens, 2013) and up to 1 μg/L in groundwater (Naseem *et al*., 2014). The important releases of Cd to the biosphere can be discussed as natural and anthropogenic activities (Perera *et al*., 2015). Natural emissions are mainly due to mobilization of naturally occurring Cd from the earth's crust and mantle; e.g., volcanic activity and weathering of rocks (ATSDR, 2012; UNEP, 2010). Anthropogenic releases on the other hand, include non-ferrous metal production, fossil fuel combustion, phosphate fertilizer manufacturing, iron, steel, and cement production, road dust, and municipal and sewage sludge incineration (ATSDR, 2012; Merkel and Sperling, 1998; Pacyna and Pacyna, 2001; UNEP, 2010).

1.4.Cadmium availability and speciation

Availability of Cd in aquatic systems is governed by its speciation. Generally, the chemical speciation of metals in aquatic systems is dependent on the specific physical/chemical factors that prevail in the local environments (Deb and Fukushima, 1999). In aqueous solutions Cd generally occurs as the divalent Cd^{2+} cation (Smolders and Mertens, 2013). The pH of the solution influences Cd mobility due to metal hydrolysis, ion-pair formation, solubility of organic matter, surface charge of oxy-hydroxides, and organic matter and clay edges. Depending on its composition, 55 to 90 % of the total soluble Cd in groundwater is present as divalent Cd^{2+} ions, while the remaining Cd is present as organic and inorganic complexes like. Examples of such complexes may include - $CdCl^{+}$, $CdCl_2^{0}$, $CdCl_3^{-}$, $Cd(SO_4)_2^{2-}$, $CdSO_4^{0}$, $CdHCO_3^{-}$, $CdCO_3^{0}$, $Cd(CO_3)_2^{2-}$, $CdOH^{+}$, $Cd(OH)_2^{0}$, $Cd(OH)_3^{-}$, Cd_2OH^{3+}, $CdNO_3^{+}$ (Baun and Christensen, 2004; Krishnamurti and Naidu, 2003; Merkel and Sperling, 1998; Sauve *et al*., 2000; Wilkin, 2007). Retrospectively, the solubility and mobility of Cd in aquatic environments is controlled by pH, concentration of dissolved organic and inorganic carbon, and the presence of clay and oxy-hydroxides, such as Fe, Mn, and Al (Anderson and Christensen, 1988; Appel and Ma, 2002; Gardiner, 1974; Krishnamurti and Naidu, 2003; Lin *et al*., 2016; Onyatta and Huang, 2006).

1.5.Cadmium exposure and effects on fish health

Literature is replete with studies on cadmium induced negative health effects on exposed fish, either in free range or in aquaculture facilities. Emerging evidences show deleterious effects on almost every vital system in fish, including reproductive, endocrine, neural,

cardiovascular, digestive, excretory, musculoskeletal systems. Reported effects have been shown to be amplified by duration of exposure, as well as dosage. El-Ebiary *et al*. (2013) reported a number of cadmium induced impairments on reproductive performance of red tilapia fish. A dose of 1 mg Cd g^{-1} inhibited spawning of females and impaired ovarian development. Separation of follicular membrane from underlying ooplasm and abnormal vacuolization of ova were observed. Also in males, this dose caused degeneration of spermatogenic elements, fibrosis of lobule walls and blood infiltration, significantly decreased sperm number and sperm motility. Cd^{2+} bioaccumulation in fish disrupts endocrine processes especially those involved in synthesis, release and metabolism of hormones (Thomas, 1993). For example, estradiol levels were significantly increased in dissolved Cd exposed female tilapia fish, and also down-regulated vitellogenin expression (Luo *et al*., 2015). The authors concluded that Cd exposures affected gonadal development by altering steroid hormone levels and sex-related gene expressions in tilapia. In the brain of exposed Zebrafish, Cd significantly abolished the estradiol-stimulated transcriptional response of the reporter gene for the three estrogen receptor (ER) subtypes in ER-negative glial cell line (U251-MG) cells, and clearly inhibited the estradiol-induction of aromatase-B in radial glial cells of Zebrafish embryos. These inhibitory effects were accompanied with a significant down-regulation of the expression of *esr1*, *esr2a*, *esr2b* and *cyp19a1b* genes compared to the estradiol-treated group used as a positive control (Chouchene *et al*., 2016). Thus, authors concluded that Cd acted as a potent anti-estrogen *in vivo* and *in vitro*. Exposure to 5 µg Cd L^{-1} was reported to damage lateral line system neuromasts in the fish sea bass, causing behavioral alterations in fish (Faucher *et al*., 2006). Following their

findings on chronic exposure of Cd in Zebrafish, Kusch *et al*. (2007) reported that exposure to low levels of cadmium throughout development may alter neurogenesis, subsequently resulting in long-term impairment of chemical cue perception. In Cd exposed juvenile Coho salmon fish, extensive loss of olfaction accompanied by histological injury to the olfactory epithelium was reported (Williams and Gallagher, 2013). In the same study, persistent behavioral deficits, histological injury and altered expression of a subset of olfactory biomarkers were still evident in the Coho salmon, even after a 16-day depuration in clean water. Olfactory impairment has also been reported to lead to disrupted chemosensation (Williams *et al*., 2016). In a different study, Cd also impaired olfaction in Zebrafish, thereby disrupting the anti-predator response, which is crucial for the survival of individuals (Volz *et al*., 2020). Cadmium induced genotoxic effects in the form of genomic instability, as a potent mutagen, and working to counteract the actions of DNA repair systems as well as inducing formation of aberrant nuclei have been reported (Cavas *et al*., 2005). *In vivo* genotoxicity and cytotoxicity assessment in $CdCl_2$ exposed *Labeo rohita* showed induced micronuclei, nuclear abnormalities such as nuclear bud, binucleates, lobed, notched and vacuolated nuclei (Jindal and Verma, 2015). Cytoplasmic abnormalities like echinocytes, acanthocytes, notched, microcytes and cells with vacuolated cytoplasm were also reported in same study. The effects observed in an organism following exposure to a heavy metal toxicant generally depend on its bioavailability within the organism.

1.6.Cadmium exposure and toxicokinetics

1.6.1. Bioavailability:

The fraction of a water borne heavy metal with the potential to bioconcentrate or accumulate within biological tissues is referred to as its bioavailability. Heavy metal bioavailability is controlled by – the organism's biology (metal assimilation efficiency, feeding strategy, size or age, reproductive stage); metal geochemistry (distribution in water and sediment, suspended matters, and metal speciation) (Roosa *et al*., 2016; Griscom and Fisher, 2004); and physical and chemical factors (temperature, salinity, pH, ionic strength, concentration of dissolved organic carbon, total suspended solids) (Bonnail *et al*., 2016). On the other hand, the actual toxicity of the metal is governed by metal speciation, presence of organic or inorganic complexes, pH, temperature, salinity, and redox conditions (Bonnail *et al*., 2016).

1.6.2. Uptake and sequestration in fish

In fish, the primary sites for uptake of waterborne heavy metals are through their gills and skin (Ayyat *et al*., 2020). This involves transfer of metals to the circulatory system through the epithelial cells present in the 'entry points' of the metals. Epithelial cells constitute a protective barrier against the external environment, but also serve as exchange interfaces with the outside world (Tsukita *et al*., 2001). This transfer across the epithelial cells consists of three phases i) uptake by the apical membrane (the interface with the external environment; ii) movement through the cell and interaction with intracellular ligands and; iii) efflux across the basolateral membrane (the interface with the circulatory system) (Foulkes, 1991). At the epithelial interphase, a cascade of cellular signaling events direct

the response of the cell appropriately. There is exchange of signals from the extracellular signaling molecules, also called first messengers (e.g. hormones or neurotransmitters like epinephrine, growth hormone, and serotonin) to the intracellular signaling molecules, also called second messengers (e.g. cyclic AMP, cyclic GMP, inositol triphosphate, diacylglycerol, and calcium). Second messengers are the key distributors of an external signal. They are released into the cytosol as a consequence of receptor activation, and are responsible for affecting a wide variety of intracellular enzymes, ion channels and transporters (Waller and Sampson, 2018). They activate intracellular signaling pathways which amplify the signal and culminate with the activation or inhibition of transcription factors, inducing a cellular response (González-Espinosa and Guzmán-Mejía, 2014). This cascade of cellular signaling is discretely regulated, and constitutes a core part of heavy metal regulation and homeostasis.

Experimental results indicate that cadmium is transported across the membrane system either through channels or by protein transporters used by some other essential metals (Zhou *et al*., 2015). For example, cadmium may enter the apical membrane of intestinal epithelial cells through the divalent metal transporter 1 (DMT1), also known as divalent cation transporter 1 (DCT1). Some zinc transporters such as ZIP1, ZIP2, ZIP8, and ZIP14 can also transport cadmium into cells (Fujishiro *et al*., 2012). Cadmium also form cadmium-protein complexes which enter cells via receptor-mediated endocytosis, example with multidrug resistant protein 1 (MRP1), cystic fibrosis trans-membrane conductance regulator (CFTR), metallothionein (MT), megalin, and cubulin (Zhou *et al*., 2015). In electrogenic cells, cadmium permeates into cells through L- or N-type voltage-dependent

calcium channels (VDCCs) (Leslie *et al*., 2006). In non-electrogenic cells, cadmium permeates into cells through the store-operated Ca^{2+} channels (SOCs) (Olivi and Bressler, 2000). Cadmium can also permeate the cells through the human transient receptor potential channel vanilloid 6 (hTRPV6) (Kovacs *et al*., 2011), which is a calcium-selective channel (Zhou *et al*., 2015). Following uptake by the various described channels, the association and nature of Cd with biomolecules then deserves attention. These associations are complex and involve biometals such as zinc (Zn) and calcium (Ca), amongst others (Ninomiya *et al*., 1993; Grosicki and Doman´ ska, 1997; Brzo´ ska and Moniuszko-Jakoniuk, 1998; Peraza *et al*., 1998; Tandon *et al*., 1994).

1.6.3. Interaction with zinc (a transition metal ion)

Zinc is the second most important trace element (after iron) essential for all living organisms. It exists as a divalent cation (Zn^{2+}). Zn^{2+} is involved in a variety of biological processes, as signaling, structural, catalytic, and intracellular and intercellular component. Zinc is functional as a signaling mediator, leading to the concept that zinc is "the calcium of the 21st century" (Frederickson *et al*., 2005). The signaling functions of zinc occur by increases in Zn^{2+} concentrations triggered by stimuli. Zinc-activated signaling is associated with pathophysiological functions (Fukada *et al*., 2011; Hirano *et al*., 2008) and therefore has therapeutic potential. Zinc plays crucial signaling roles as a second messenger in the cytosol (Fukada *et al*., 2011; Hirano *et al*., 2008), where zinc signaling is caused by zinc influx, which originates from extracellular sites and from intracellular organelles.

By association, Cd and Zn share many similarities. Cd is a by-product obtained during zinc mining. Cd^{2+} and Zn^{2+} ions share similar electronic configuration on their outermost shells, each having a valence of II as their stable form. However, Zn is more stable in its divalent form, and also redox neutral, unlike Cd. In biological systems Cd and Zn are linked to macromolecules, primarily through sulfur (S), oxygen (O) and nitrogen (N) and interact readily with S-, O- and N donors. They bind preferentially to the same proteins —albumin in the bloodstream and metallothionein (MT) and other proteins in tissues (Brzóska and Jakoniuk, 2001).

Although both metals have a high affinity to biological structures (proteins, enzymes) containing –SH (sulphydryl) groups, the affinity of Cd to S-ligands as well as to N-donors is greater than that of Zn (Jacobson and Turner, 1980; Jones and Cherian, 1990). As a result, Cd preferentially displaces Zn in a number of biological processes (Gachot and Poujeol, 1992; Endo *et al*., 1996, 1997). By displacing Zn, Cd interferes with Zn absorption, distribution into tissues, and transport into cells or several intracellular structures (Brzóska and Jakoniuk, 2001). This interference in homeostatic activities of Zn is responsible for the toxic effects of Cd, including cellular production of DNA, RNA and protein (Sunderman and Barber, 1988). As may be seen already, deleterious effects of Cd in fish can be overwhelming, affecting fish at molecular, biochemical, and physiological levels.

1.6.4. Interaction with calcium (an essential alkaline earth metal)

Ca^{2+} is a key signaling ion involved in many different intracellular and extracellular processes ranging from synaptic activity to cell-cell communication and adhesion (Marambaud *et al*., 2009). Ca^{2+} is responsible for triggering many responses to external stress conditions (Putney, 2005) and in regulating enzyme activity and permeability of ion channels (Ali *et al*., 2016). Ca^{2+} homeostasis disruption implicates several mechanisms, such as alterations of calcium buffering capacities, deregulation of calcium channel activities, or excitotoxicity (Marambaud *et al*., 2009). The concentration of Ca^{2+} in the cytoplasm is normally kept at a very low level, about 10^{-7}M, while that in the extracellular medium is of the order of 10^{-3}M. Inside the cell, there is a Ca^{2+} storage site, which is a membrane-bound organella that strongly accumulates Ca^{2+} to such an extent that its luminal Ca^{2+} concentration reaches millimolar level. Thus, there is a huge concentration gradient of Ca^{2+} across the surface membrane and membrane of the intracellular Ca^{2+} store. Some kinds of stimulation of the cell trigger a flow of Ca^{2+} from either the extracellular space or the intracellular Ca^{2+} store into the cytoplasm, usually by opening Ca^{2+} channels present in the boundary membrane to allow a passive Ca^{2+} movement down the electrochemical potential gradient. Ca^{2+} thus mobilized into the cytoplasm causes the response of the cell to the stimulus. When the stimulus ceases, Ca^{2+} is extruded back to the extracellular space or re-accumulated to the intracellular Ca^{2+} store to terminate the response. Active transport is necessary in this process because Ca^{2+} must move against the large electrochemical potential gradient. Thus, Ca^{2+} plays a role as a second messenger, which, in response to a stimulus delivered by a first messenger from outside, transmits the message of arrival of the

stimulus to the intracellular system that carries out the response of the cell to the stimulus. Ca^{2+} is one of the most important second messengers (Endo, 2006).

Cd^{2+} structurally resembles Ca^{2+}, and participates in a number of Ca^{2+} dependent pathways (Choong *et al*., 2014), a process referred to as "ionic and molecular mimicry" (Vesey, 2010). Cd^{2+} interacts with receptors and ion channels on the cell surface, and with the intracellular estrogen receptor where it binds competitively to residues shared by Ca^{2+}. It increases cytosolic Ca^{2+} through several mechanisms, but also decreases transcript levels of some Ca^{2+}-transporter genes. It initiates mitochondrial apoptotic pathways, and activates calpains, contributing to mitochondria-independent apoptosis (Choong *et al*., 2014). These interferences by Cd^{2+} therefore disrupt the homeostatic roles of Ca^{2+}, leading to dyshomeostasis and toxic effects. Other interfering roles of Cd^{2+} on Ca^{2+} include inhibition of the plasma membrane Ca^{2+}-ATPase (PMCA) thereby blocking Ca^{2+} efflux (Akerman *et al*., 1985), preventing calcium entry into the endoplasmic reticulum and Golgi apparatus by inhibiting sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) or secretory pathway Ca^{2+}-ATPase (SPCA) activities (Biagioli *et al*., 2008; Ohrvik *et al*., 2011). From the foregoing, it is pertinent that negative effects of cadmium, or heavy metals as a whole, is mediated primarily by interference with metabolic intracellular activities. Accordingly, there is proliferation and buildup of endogenously generated radical species and other non-radical reactive derivatives, which otherwise are regulated during homeostasis. These reactive species are generally called oxidants.

1.7. Reactive species, free radicals, and generation of oxidative stress

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the terms collectively describing free radicals and other non-radical reactive derivatives also called oxidants (Pham-Huy *et al*., 2008). Reactive species and free radicals are unstable and highly reactive molecules formed as intermediates or by products of metabolic reactions. Oxygen derived reactive species, otherwise known as reactive oxygen species (ROS) include superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and oxides of nitrogen ($NO^{\bullet}$, $NO_2^{\bullet}$). Due to their molecular instability (e.g., unpaired electron), they promote oxidation reactions with other molecules, such as proteins, lipids, and DNA, in order to become stabilized (Fisher-Wellman and Bloomer, 2009; Halliwell and Gutteridge, 2007). It has also been suggested that free radicals play many beneficial roles in the body and are necessary for many physiological functions of living organisms (Sies, 1991; Afanas'ev, 2007). They are required for many important signaling reactions (Mittler, 2017). For example, hydrogen peroxide and peroxynitrite, in particular, have been implicated in a considerable number of cellular signaling cascades (Wolin, 1996; Rhee, 1999; Levonen *et al*., 2001). ROS are also imperative for redox homeostasis, as well as proper function in the cardiovascular system, and immune system (Patel *et al*., 2017). They are produced by immune cells—neutrophils and macrophages—during the process of respiratory burst in order to eliminate antigens (Freitas *et al*., 2010). At low to moderate concentrations, ROS play important roles in the body by regulating cellular processes. However at high concentrations, they can adversely modify important biomolecules such as cellular lipids, proteins, and DNA, thus leading to oxidative stress (Liemburg-Apers *et al*., 2015),

impairment of diverse cellular functions (Gomes *et al*., 2012), and ultimately tissue and organ injury or failure (Oter *et al*., 2012). On the other hand, if the level is too low, reductive stresses occur and can also cause pathologies ranging from cancer to cardiomyopathy (Liou and Storz, 2010).

1.8.Antioxidants and ameliorating roles in oxidative stress

As their name imply, antioxidants oppose the activities of oxidants. Antioxidants are a broad group of compounds which constitute the first line of defense against free radical damage thus are essential for maintaining optimum health and well-being. They are protective agents, capable of stabilizing or deactivating free radicals before they attack cells (Bahorun *et al*., 2006). Antioxidant molecules inhibit, decrease, delay, or completely scavenge free radicals and oxidants, and protect the body from oxidative damage (Lobo *et al*., 2010). Living organisms possess multilevel and complicated antioxidant systems operating to prevent ROS formation, eliminate ROS and ROS-modified molecules, or minimize their negative effects (Lushchak, 2016). A complex network of antioxidant metabolites and enzymes work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids (Sies, 1997). Antioxidants may be broadly categorized into enzymatic and non-enzymatic antioxidants (Fig. 1.). Of the enzymatic antioxidants, the primary and secondary are endogenously generated, in addition to a few others like uric acid, vitamin A (retinol), glutathione, and coenzyme Q10. A majority of the non-enzymatic antioxidants are of exogenous origin, and may be found in plant dietary sources. For fish, most of these are acquired from their diet but the tripeptide glutathione

(c-glutamyl-cysteinyl-glycine, GSH) is synthesized by most aerobic organisms and used to control ROS levels either via direct interaction with them or by serving as a cofactor for ROS-detoxifying enzymes (Marí *et al*. 2009; Lushchak, 2012).

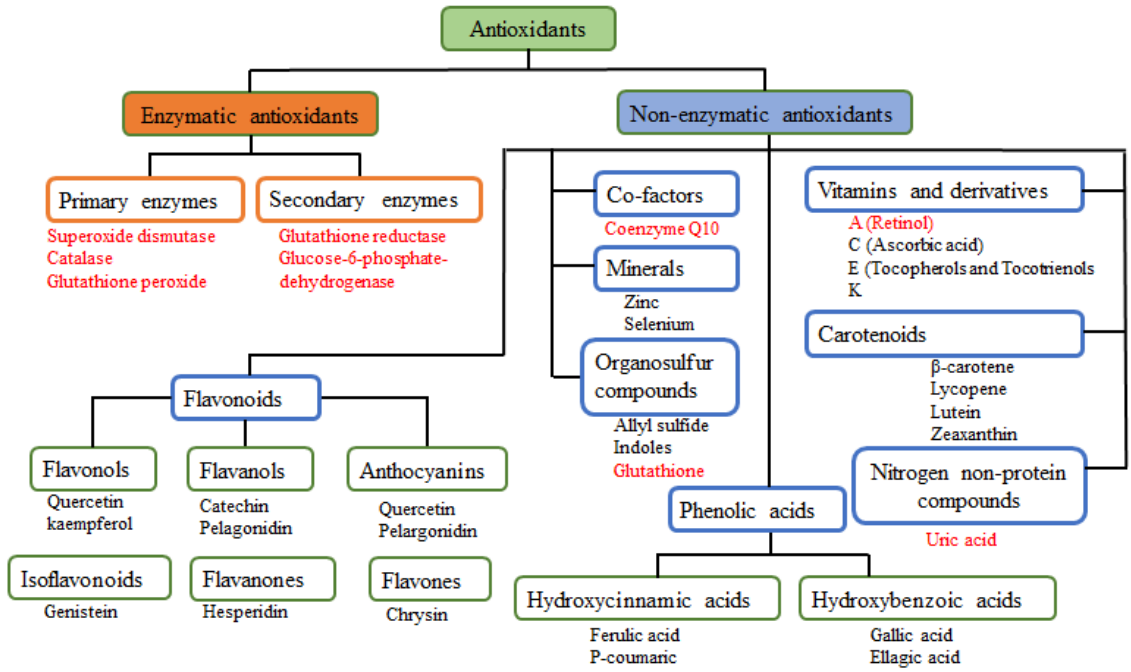

Fig. 1. Natural antioxidants separated in classes. Black words outside box represent exogenous antioxidants, while red ones represent endogenous antioxidants. Adapted from Pietta, (2000); Ratnam *et al*. (2006); and Godman *et al*. (2011).

1.9.Mode of action of antioxidants

In principle, defense strategies of antioxidants include three levels of protection - prevention, interception, and repair (Sies, 1993). Prevention which is the first line of defense against reactive oxygen species protects against their formation. Here, preventive antioxidants act by quenching of $O_2^{\cdot-}$, decomposition of H_2O_2, and sequestration of metal

ions (Irshad and Chaudhuri, 2002). For example, SOD, a first line defense against free radicals, catalyzes the dismutation of superoxide anion radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) by reduction. The oxidant formed (H_2O_2) is transformed into water and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GPx). The selenoprotein GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. Besides hydrogen peroxide, GPx also reduces lipid or nonlipid hydroperoxides while oxidizing glutathione (GSH) (Pham-Huy *et al*., 2008; Bahorun *et al*., 2006b; Dröge, 2002; Willcox *et al*., 2004; Pacher *et al*., 2007; Genestra, 2007; Halliwell, 2007; Young and Woodside, 2001).

Also referred to as scavenging antioxidants, the second line of defense scavenges active radicals to inhibit chain initiation and break chain propagation reactions. They neutralize free radicals by donating electron to them, and in the process become free radicals themselves but of lesser damaging effects. These 'new radicals' are easily neutralized and made completely harmless by other antioxidants in this group (Ighodaro and Akinloye, 2018). Examples include ascorbic acid, uric acid, glutathione, which are hydrophilic and alpha tocopherol (vitamin E) and ubiquinol which are lipophilic in nature. This group of antioxidants acts as 'interceptors'. The third line defense antioxidants are *de novo* antioxidants. They swing into action following free radical to biomolecules. They effect repair to damaged biomolecules such as proteins, lipids, and DNA. They recognize, breakdown and remove oxidized or damaged proteins, DNA and lipids, to prevent their accumulation which can be toxic to body tissues. Examples include the DNA repair enzyme

systems (polymerases, glycosylases and nucleases), proteolytic enzymes (proteinases, proteases and peptidases) which are located both in cytosol and mitochondria of mammalian cells (Ighodaro and Akinloye, 2018). The action of fourth line defense antioxidants prevents the formation or reaction of free radicals through special adaptation mechanism signal. The signal generated from the free radical formed induces the formation and transport of an appropriate antioxidant to the right site (Niki, 1993). The third and fourth line antioxidant defense systems act to effect repairs of damaged biomolecules. Kalam *et al*. (2012) aptly describes the mechanism of action of antioxidants (Fig. 2).

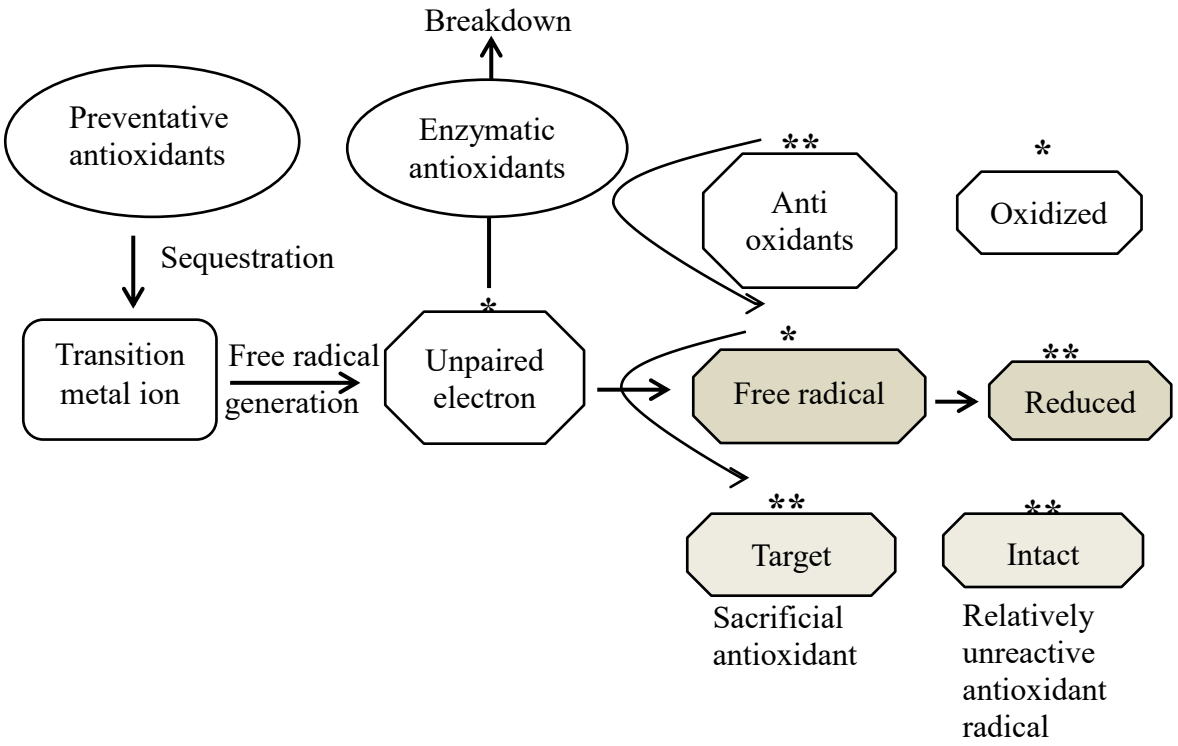

Fig. 2. Mechanism of action of antioxidants. The symbol ⁎ represent single or unpaired electron, while ⁎⁎ represent paired electrons.

1.10. Plants derived compounds and roles in toxicity amelioration

From previous decades, extensive research attention has been focused on food pants and their medicinal values. The use of plants as food or medicine can be difficult to differentiate (Júnior *et al*., 2015; Ferreira *et al*., 2016; Jennings *et al*., 2015; Murray and Pizzorno, 2012), and the overlap between these two categories of uses is multidimensional (Leonti, 2014). Some plants may be used only as a tonic or functional food to improve health instead of to treat a specific disease, but some others may be used as both food and medicine, even with the same organ parts and application methods (Jennings *et al*., 2015; Jiang and Quave, 2013; Pieroni and Quave, 2006). This important uses has accompanied every clime and culture from their respective historical antecedents, and has remained relevant even till this day. They have been extensively employed in the treatment of chronic diseases and the improvement of physical fitness, especially in some developing countries and minority tribal areas (Jiang and Quave, 2013; Hong *et al*., 2015). The main compounds found in plants correspond to four major biochemical classes: Polyphenols, terpenes, glycosides and alkaloids. Plants synthesize these compounds for a variety of purposes, including protection of the plant against fungi and bacteria, defense against insects and attraction of pollinators and dispersal agents to favor the dispersion of seeds and pollens (Nieto, 2020). In recent decades however, the antioxidant potentials of plants have been explored, especially in the prevalence of heavy metal induced oxidative stress. These natural antioxidants from plant materials are mainly polyphenols (phenolic acids, flavonoids, anthocyanins, lignans and stilbenes), carotenoids (xanthophylls and carotenes) and vitamins (vitamin E and C) (Baiano and Del Nobile, 2016; Manach *et al*., 2004). Plant amino acids (AAs) have also

been shown to poses important antioxidant properties (Dash and Ghosh, 2018; Liu *et al*., 2018). They are considered as synergistic antioxidants because they enhance the effects of other antioxidants, regenerate oxidized primary antioxidants, inactivate reactive oxygen species (ROS), and scavenge free radicals (Marcuse, 1960). Antioxidants from food and medicinal plants, such as fruits, vegetables, cereals, mushrooms, beverages, flowers, spices and traditional medicinal herbs (Deng *et al*., 2013; Cai *et al*., 2004; Shan *et al*., 2005; Fu *et al*., 2011) have also been extensively studied. This study considered garlic, propolis (from honey bee), and wakame due to their rich antioxidative potentials. For perspective, their antioxidative merits and current studies on them shall be discussed. A comparative assessment on the basis of their common antioxidant contents such as polyphenols, vitamins, and amino acids is also considered.

1.10.1. Garlic (*Allium sativum*)

Fig. 3. *Allium sativum* cloves

Garlic belongs to the onion family, alongside shallots, leeks, and chives. Its taxonomical classification is shown in table 1.

Table 1. Taxonomic hierarchy of garlic (*Allium sativum*)

Domain	Eukarya (multicellular)
Kingdom	Plantae (plants)
Subkingdom	Tracheobionta (vascular plants)
Superdivision	Spermatophyta (seed plants)
Division	Magnoliophyta (flowering plants)
Class	Liliopsida (monocotyledons)
Subclass	Liliidae
Order	Liliales
Family	Liliaceae (lily family)
Genus	*Allium* L. (onion)
Species	*Allium sativum* L. (cultivated garlic)

Garlic (*Allium sativum* L.) (Fig. 3) is an underground bulbous crop common in almost all parts of the globe. It is said to be the oldest cultivated herb, with its origin linked to central Asia (Fenwick and Hanley, 1985; Block, 2010), and northeastern Iran (Block, 2010). Its culinary use dates several thousand years of human history. For example, in the bible, it was known to ancient Egyptians and Israelites (Numbers 11:5). Garlic has acquired a reputation in different traditions as a prophylactic as well as therapeutic medicinal plant (Bayen *et al*., 2014). There is appreciable epidemiologic evidence that demonstrates therapeutic and preventive roles for garlic (Bayen *et al*., 2014). The nutritional composition of garlic is shown in Table 2.

Table 2. Nutritional composition of garlic

Nutritional composition	Amount	Reference
Water activity	0.98	Toledano-Medina *et al*. (2016)
Water-soluble sugar	72.29% DM	Sasaki *et al*. (2007)
Reducing sugar	5.9 ± 0.8 mg/g DM	Martinez-Casas *et al*. (2017)
Lipid	1.8 ± 0.1 mg/g FM	Choi *et al*. (2008)
Protein	8.4% FM	Sasaki *et al*. (2007)
Amino acid	19.43 ± 0.01 mg /g FM	Lu, 2017
SAC	0.02 ± 0.00 mg /g DM	Bae *et al*. (2014)
Organic acid	16.68 ± 0.61 mg /g DM	Zhang, li *et al*. (2016)
Allicin	11.28 ± 0.22 mg /g FM	Zhang *et al*. (2015)
Polyphenol	38.87 ± 4.53 mg /g DM	Choi *et al*. (2014)
Melanoidin	< 0.1 OD FM	Yuan *et al*. (2018)
Vitamin	6.92 ± 0.02 mg /g DM	Kim *et al*. (2013)
Mineral	11.74 ± 0.02 mg /g DM	Kang (2016)
SOD	12.96 mg /g FM	He *et al*. (2008)

Garlic is rich in proteins and polyphenols (Cardelle *et al*., 2010). Moreover, it is an abundant source of certain vitamins like vitamin A, vitamin B_6 and B_1 and vitamin C (Neeraj *et al*., 2014; Kim *et al*., 2013). Garlic contains at least 33 sulfur compounds, several enzymes, 17 amino acids, and minerals such as selenium. It contains a higher concentration of sulfur compounds than any other allium species (Eigurg *et al*., 2015). Organo-sulfur compounds are well known for their chemo-preventive activities which increase with the polyphenols, carotenoids, isothiocyanates, and allyl sulfides, lycopene (Eksi *et al*., 2019). Gupta *et al*. (2008) reported the prospects of garlic to prevent heavy metal induced alterations in lipid profile of male albino rats. Alterations in lipid profile are implicated in lipid peroxidation. Lipid peroxidation is considered as the main molecular mechanisms that lead to oxidative damage to cell structures and in the toxicity process that leads to cell death (Repetto *et al*., 2012). Garlic has antioxidant properties (Rahman, 2003), which is able to mitigate against the propagation of reactive oxygen species, ROS which induces lipid peroxidation. In pathological situations, the reactive oxygen and nitrogen species are generated at higher than normal rates, and as a consequence, lipid peroxidation occurs (Repetto *et al*., 2012). Addition of garlic to fish diets increased the erythrocyte number, hemoglobin content, hematocrit, leukocyte number, and thrombocyte number (Martins *et al*., 2002). Significant changes in serum total protein and globulin was also reported in rainbow trout following garlic diet supplementation (Nya and Austin, 2009). A three month garlic oil treatment at 250 mg/kg diet demonstrated beneficial effects in improving lipid profile of copper and zinc exposed *Oreochromis niloticus* (Metwally, 2009). Garlic offered protective effects against Cd induced toxicity in sea bass head, liver and kidney tissues

(Mosbaha *et al*., 2017). Garlic also decreased the accumulation and toxic effects of lead (Pb) and Cd in the blood, liver and kidney of male albino rats (Tandon *et al*., 2001).

The major biologically active component of garlic is allicin. Fresh garlic clove contains allin (an amino acid) and allinase (an enzyme). When fresh garlic is chopped, the enzyme allinase reacts with the amino acid allin to form allicin, while giving off the characteristic off flavor which garlic is known for. Allicin rapidly breaks down to form a variety of organosulfur compounds (Block, 1985). It is thought that a single garlic clove has about 5 mg to 18 mg of allicin. Some reports posit the therapeutic use of garlic to be strongly linked to its allicin content. Yet, allicin is also known to be highly unstable (Suciu *et al*., 2016) and degrades rapidly in the acidic environment of the stomach (Prati *et al*., 2014), and may be limiting in its therapeutic use considerations. On the other hand, the rich bioactive phenolic compounds in garlic (Lanzotti, 2006; Corzo-Martínez *et al*., 2007) has been reported to exhibit significant antioxidant activity (Park *et al*., 2009), scavenge reactive oxygen species (ROS) (Queiroz *et al*., 2009) and protect against oxidative DNA damage (Park *et al*., 2009). From the hindsight of reported studies, the successes with therapeutic use of garlic formed a motivation for its use in the present study. In the context of the current study, use of garlic in amelioration of cadmium bioconcentration and induced toxicity effects in surviving fish following water remediation is yet to be addressed.

1.10.2. Propolis (*Apis*)

Fig. 4. Propolis from honey bee

Propolis is a natural resinous sticky product composed by honey bees to be used as a sealant and sterilant in their nests (Sforcin *et al*., 2000). Its rich biochemical profile include such compounds as polyphenols, amino acids (Eroglu *e al*., 2016), vitamins (Usman *et al*., 2016) and several other important inorganic compounds (Table 3). These compounds are reputable for their biological and pharmacological properties. Hence, propolis has been used as a natural immune and growth stimulant (Chu, 2006; Abd-El-Rhman, 2009; Zhang *et al*., 2009; Talas and Gulhan, 2009; Meurer *et al*., 2009; Deng *et al*., 2011; Bae *et al*., 2012; Šegvić-Bubić *et al*., 2013), hepato-protection and detoxification process (Mani *et al*., 2006; Castaldo and Capasso, 2002; Jasprica *et al*., 2007; Kanbura *et al*., 2009; Šegvić-Bubić *et al*., 2013).

Table 3. Nutritional composition of propolis

Nutritional composition	Amount	Reference
Moisture level	17.11 % DM	Usman *et al*. (2016)
Carbohydrate	21.94 % DM	Usman *et al*. (2016)
Quercetin	0.17 mg /g	Usman *et al*. (2016)
Total phenol	1.93 mg /g	Usman *et al*. (2016)
Total vitamins	0.37 mg /g	Usman *et al*. (2016)
Calcium	0.29 mg /g	Usman *et al*. (2016)
Magnesium	0.26 mg /g	Usman *et al*. (2016)
Zinc	0.00071 mg /g	Usman *et al*. (2016)
Total amino acid	27.65 mg /g	Eroglu *et al*. (2016)

Fish specific studies with propolis have demonstrated its ameliorative roles in various toxicant induced oxidative stress and toxic effects (Hamed and Abdel-Tawwab, 2017; Kakoolaki *et al*., 2013; Talas *et al*., 2014). For example, in addition to improving fish fillet quality, propolis-enriched diet ameliorated toxic effects induced by potassium dichromate in Nile tilapia fish (El-Borai *et al*., 2018). Although numerous pharmacological properties of propolis have been highlighted, there is paucity of information with fish studies. The highlighted beneficial roles therefore give impetus for its use in the context of this study, as no study has assessed its toxicity amelioration and recovery enhancement roles during recovery in Cd exposed fish.

1.10.3. Wakame

Fig. 5. Wakame (*Undaria pinnatifida*)

Table 4. Taxonomical hierarchy of wakame (*Undaria pinnatifida*)

Domain	Eukaryota
Kingdom	Plantae
Phylum	Phaeophyta
Class	Phaeophyceae
Order	Laminariales
Genus	Undaria
Species	*U. pinnatifida*

Wakame (*Undaria pinnatifida*), a popular species of edible seaweed in Japan is known to contain valuable pharmacological compounds such as large quantities of proteins (Suetsuna *et al*., 2004), polysaccharide (Hu *et al*., 2010), fucoxanthin (Woo *et al*., 2009) and to be rich

polyphenols (Wang *et al*., 2018), amino acids (Taboada *et al*., 2013), and various kinds of macro and trace elements (Dawczynski *et al*., 2007). Nutritional composition of wakame is shown in table 5.

Table 5. Nutritional composition of wakame

Nutritional composition	Amount (mg /g)	Reference
Total amino acids (mg /g of protein)	51.88 ± 9.73	Taboada *et al*. (2013)
Calcium	6.93 ± 0.60	Taboada *et al*. (2013)
Phosphorus	10.70 ± 0.70	Taboada *et al*. (2013)
Iron	0.08 ± 0.00	Taboada *et al*. (2013)
Magnesium	6.30 ± 0.14	Taboada *et al*. (2013)
Zinc	0.04 ± 0.0	Taboada *et al*. (2013)
Iodine	0.10 ± 0.0	Taboada *et al*. (2013)
Total polyphenol (mg /g DM)	25.12 ± 1.02	Moreira *et al*. (2014)
Total vitamin (mg /g DM)	4.78 ± 0.86	Taboada *et al*. (2013)

Studies with *U. pinnatifida* have reported radical scavenging properties of the seaweed (Je *et* al., 2009; Kim *et al*., 2015; Rafiquzzaman *et al*., 2013). Moreover, fucoidan extracted from wakame was shown to have anti-inflammatory (Ale *et al*., 2011; Li *et al*., 2008), immunomodulatory (Kim and Joo, 2008), antitumor (Costa *et al*., 2011), and anticoagulation activities (Kim *et al*., 2010). Seaweeds generally have shown beneficial effects or absence of adverse effects at low inclusion levels of up to 10% for the majority of the seaweeds and fish species tested (Valente *et al*., 2005; Rajaura, 2015; Chandini *et al*.,

2008). Zhang *et al*. (2014) reviewed pharmacological properties of wakame in other terrestrial experimental models (Table 6). However presently, there is a dearth of information on *U. pinnatifida* and fish specific studies. Against this backdrop, this study explored the beneficial prospects of wakame in Cd induced depuration and toxicity amelioration in medaka fish juveniles.

Table 6. Main pharmacological properties of wakame (*Undaria pinnatifida*)

Pharmacological property	References
Antihypertensive *in vitro*	Sato *et al*., 2002
SHRs	Sato *et al*., 2002; Suetsuna *et al*., 2004
Anti-inflammatory and rats pro-inflammator	Khan *et al*., 2007
Antioxidant activity *in vitro*	Hu *et al*., 2010
Innate immune stimulating activity *in vitro*	Fujiyuki *et al*., 2012
Antitumor activity *in vitro*	Li *et al*., 2008
Antiviral activity *in vitro*	Harden *et al*., 2009; Carlucci *et al*., 1999
Anti-influenza activity *in vitro*	Itoh *et al*., 1995
Murine models	Hayashi *et al*., 2007
Anti-coagulating activity	Kim *et al*., 2009
Anticancer activity *in vivo* and *in vitro*	Yang *et al*., 2008
Anti-obesity activity *in vivo* and *in vitro*	Maeda *et al*., 2007
Anti-diabetic effects mouse model	Maeda *et al*., 2007

1.11. Experimental model: Japanese medaka fish (*Oryzias latipes*)

The Japanese medaka fish (*Oryzias latipes*) (Fig. 6) is a small freshwater fish of the family Adrianichthyidae in the order Beloniformes (Table 7).

Table 7. Taxonomic classification of Japanese medaka fish (*Oryzias latipes*)

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Superclass	Gnathostomata; Pisces
Class	Actinopterygii
Order	Beloniformes
Family	Adrianichthyidae
Subfamily	Oryziinae
Genus	Oryzias
Species	*Oryzias latipes*

It is closely related to other members of the superorder Acanthopterygii of ray-finned fish such as puffer fish (tetraodon and fugu), stickleback and killifish (Kirchmaier *et al*., 2015) (Fig. 7). Medaka is native to Taiwan, Korea, China, and Japan. In Japan it inhabits small rivers, creeks, and rice paddies on all main islands, with the exception of Hokkaido (Kirchmaier *et al*., 2015). It is a euryhaline species and can also live in brackish water (Inoue and Takei, 2002). As a resident of a temperate zone, medaka can tolerate a wide range of temperatures (4–40°C) both as adult and embryo (Sampetrean *et al*., 2009). Thus, in the laboratory, temperature can be used to control the developmental speed without adverse effects (Kirchmaier *et al*., 2015). Constant mating adults in the laboratory (14 hr light/10 hr dark at 25–28°C) can live up to about 12 months, but can be extended to >2

years under conditions where the fish do not mate in combination with a reduced temperature (10 hr light /14 hr dark at 19°C) (Kirchmaier *et al*., 2015). Medaka fish show seasonality in mating behavior, requiring long light phases and short dark phases for reproduction, whereas temperature has little effect on fecundity (Koger *et al.* 1999). They exhibit a distinct sexual dimorphism, hence easy to tell the males apart from females. Medaka is oviparous with transparent eggs and embryos. Embryos hatch after 7–8 days at 28°C as fully developed juvenile fish. The generation time of medaka is 8–12 weeks depending on strain and husbandry conditions.

Fig. 6. Japanese medaka fish (*Oryzias latipes*)

Japanese medaka (*Oryzias latipes*) is used in this study due to its numerous research qualities. It is a small freshwater fish that have been used for over 50 years for toxicity testing (Padilla *et al*., 2009). It serves as a model vertebrate organism in various fields of biology including development, genetics, toxicology, evolution (Sasado *et al*., 2010), and human diseases (Ishikawa, 2000; Wittbrodt *et al*., 2002). Compared with mammalian models such as mice and rats, the *Oryzias latipes* is small, cost-effective, easy to breed in

large numbers, prolific and can be confined easily. Furthermore, most experimental tools for the analysis of gene function can be applied to medaka (Furusawa, 2006). Although many reports have documented its use in toxicological studies, its welfare and recovery dynamics from Cd and other environmentally relevant heavy metals is yet to be addressed. More so, as pollution by heavy metals in aquatic systems appears to be almost inescapable to fish, it becomes crucial to examine its recuperation dynamics.

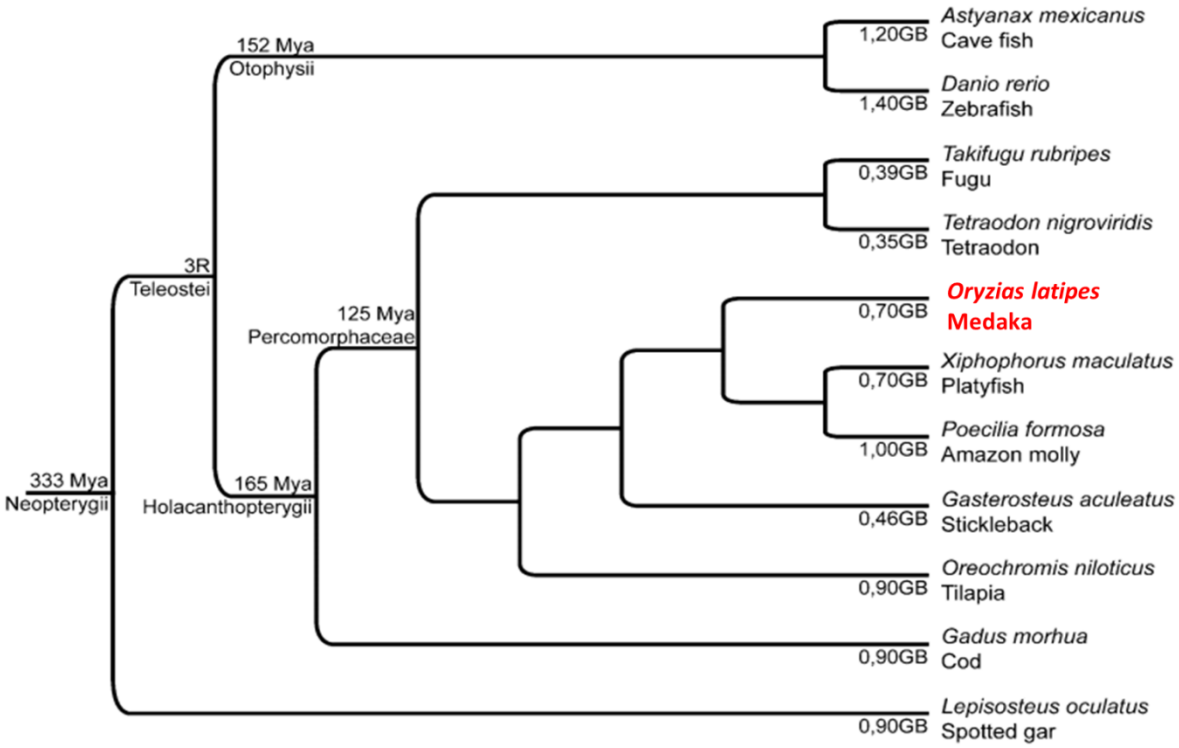

Fig. 7. Evolutionary relationship of medaka and other teleost model systems. Only teleost model systems with publicly available genome sequence data on EnsEMBL are shown. The spotted gar is an outgroup of the teleost, which did not undergo a whole-genome duplication (3R). Medaka belongs to the Percomorphaceae, the clade with most sequenced teleost genomes, making it an ideal organism for comparative genomics. For each species, the Latin and the common name as well as the genome size is shown. The species tree was calculated using the EnsEMBL compara team (http://www.ensembl.org/info/genome/compara/). Taxonomy data are available at EnsEMBL and derived from the National Center for Biotechnology Information taxonomy browser.

1.12. Problem statement

Pollution by heavy metals is almost inescapable to fish. The ubiquity, persistence, and toxicity effects of Cd on the other hand, pose great danger to fish and successive fish consumers along the food chain. Animals that accumulate cadmium in their bodies ("body burden") can be eaten by others, and so on, such that cadmium will both accumulate and biomagnify in the food chain (EPA 2000). Thus, rapid recovery of a fish species from heavy metal exposure and effects will ensure its improved performance and species viability. More so, health risks to fish consumers along successive links in the food chain will be substantially reduced. As yet however, studies in fish which bother on the amelioration of bioconcentration and toxicity effects of Cd are limited. The prospects of garlic, propolis, and wakame as supplemental dietary sources during recuperation in Cd exposed Japanese medaka fish is also yet to be fully explored.

1.13. Purpose of the study

The aim of this study was to assess the effects of garlic, propolis, and wakame supplemented diets on recuperation in Cd exposed Japanese medaka fish, taking advantage of their immune stimulating and antioxidative potentials. The specific objectives were to:

(i) Investigate Cd uptake and depuration levels in gill, liver, and muscle tissues of subjects following Cd exposure and dietary treatments, respectively;

(ii) Compare bioconcentration factor in the three dietary regimes

(iii) Investigate Cd induced toxicity levels in gill, liver, and muscle tissues of subjects, following Cd exposure and dietary treatments, respectively;

(iv) Compare magnitude of toxicity amelioration in the three dietary regimes.

1.14. Structure of dissertation

This thesis is organized into four chapters. Chapter 1 presents a general introduction and purpose of this study. It also reviews pertinent literature focusing on sources of Cd pollution in aquatic systems, exposure and tissue concentration in fishes, toxicity effects, and roles of dietary plant derived compounds in containment of Cd bioconcentration and induced toxicity. It is succeeded by chapter 2, which assesses cadmium bioconcentration and health risk in Japanese medaka (*Oryzias latipes*) under three dietary regimes. Here, a focus on cadmium biomagnification along successive links in the aquatic food chain, through whole body cadmium bioconcentration is explored. This chapter also provides insight into reduction in cadmium biomagnification risk through the respective supplemented diets. Chapter 3 explores comparative assessment of cadmium induced toxicity and recuperation dynamics in Japanese medaka (*Oryzias latipes*) under three dietary regimes. Here the major organs of direct contact with, and exposure to cadmium in fish are considered. A part of the findings of this study has also been published in the Journal of Environmental Science and Health, part A. Chapter 4 discusses in detail the findings and implications of the study, summarizes all findings of the study, and presents conclusions and recommendations for further studies.

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Chapter 2: Comparative assessment of cadmium bioconcentration and health risk in Japanese medaka under three dietary regimes

Abstract

Cadmium (Cd^{2+}) uptake and concentration within tissues of aquatic subjects is a major health risk associated with Cd exposure in aquatic environments. Carnivorous species and fish eaters along successive links in aquatic food chain are known to swallow whole organisms. Consequently, Cd tissue burden magnifies up the food chain. Considering the long half-life of Cd, the prospects of continuous exposure even after water remediation raises serious health risk concerns. In addition to their rich antioxidative merits, garlic, propolis, and wakame have been shown to contain bioactive compounds which interfere in heavy metal uptake, particularly Cd, and also depuration within tissues. Thus, the aim of this study was to comparatively assess i) Cd bioconcentration, and ii) Cd depuration in exposed medaka fish juveniles under three dietary regimes. Fish were exposed to 0.3 mg Cd /L in water for 21 days under a control diet. Surviving fish were further depurated for 21 days while being fed garlic (GD), propolis (PD), wakame (WD) supplemented diets, or medaka (control) diets (MD). Fish gills, liver, and muscles were assessed weekly for Cd bioconcentration and depuration, and metallothionein induction (MT). Results showed increased Cd bioconcentration and MT induction following Cd exposure. Following dietary

treatments however, there was significant decrease in Cd bioconcentration across tissues and an upregulation in MT expression. In conclusion, dietary supplementation supported Cd depuration and potentially reduced bioconcentration margin and associated health risks in the order GD > PD > WD > MD.

2.1. **Introduction**

Cadmium (Cd) is a toxic non-essential transition metal that poses a health risk for both humans and animals (Genchi *et al*., 2020). Like other heavy metals, Cd is persistent and highly cumulative. The biologically significant ionic form of Cd, Cd^{2+}, binds to many bio-molecules and these interactions underlie the toxicity mechanisms of Cd (Moulis, 2010). The persistence and cumulative character in biological tissues, suggests possible persistence in their effects even in the absence of further exposure. Homeostasis and natural defense mechanism can become undermined in the overwhelming presence of the metal. Moreover, fish and seafood are one of the main links between the heavy metal present in the environment and the human exposure (Verbeke *et al*., 2005; Burger and Gochfeld, 2009; Fraser *et al*., 2012). Fish and other aquatic food organisms bioconcentrate heavy metals from the aquatic environment to unsafe and toxic levels within their tissues. In turn, when they are ingested by man, man becomes exposed to the heavy metal, thereby completing the exposure link. The enumerated consequences which accompany heavy metal exposure in biological systems have necessitated research on their removal from aquatic environments, or of the mitigation of their impacts. Like other vertebrates, fish have

regulatory and defense mechanisms against xenobiotics and other noxious intruders. For example, metallothioneins (MTs) are cysteine rich metalloproteins, whose synthesis are closely related to metal exposure (Van Cleef-Toedt *et al*., 2001). The intracellular MTs bind Cd ions and form CdMT. In chronic intoxication, Cd stimulates *de novo* synthesis of MTs; it is assumed that toxicity in the cells starts when loading with Cd ions exceeds the buffering capacity of intracellular MTs (Sabolic *et al*., 2010). Riggio *et al*. (2003) also reported increase in MT of around 30-fold following exposure of Zebrafish (*Danio rerio*) to Cd.

Garlic, propolis and wakame were selected in this study based on their immune stimulating and antioxidative merits. Moreover, garlic was reported to prevent accumulation of Cd in rats, by enhancing biosynthesis of MT and glutathione (Tandon *et al*., 2001). Administration of propolis extract increased significantly the metallothionein (MT) concentration in whole body fractionated gamma irradiated rats (Nada and Azab, 2005). There appear to be no literature reports yet on MT induction in fish or other model species by wakame. Although the immune stimulating and antioxidative properties of wakame have been previously reported, it is not clear if wakame can induce MT in Cd exposed fish. Thus, this study appears to be investigating for the first time, the modulatory effects of wakame on Cd bioconcentration and toxicity in fish tissues. In addition, the protective role of garlic, propolis and wakame over tissue Cd bioconcentration and enhanced Cd depuration during recovery in fish is investigated in this study.

2.2. **Materials and methods**

2.2.1. Materials

Cd standard solution, nitric acid, and Tricaine methanesulfonate, MS-222 were bought from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan. Metallothionein (MT) and Enzyme-linked Immunosorbent Assay (ELISA) kits (Lot# 60361) were purchased from Frontier Institute Co. Ltd. (Hokkaido, Japan). All other chemicals used were of analytical grade.

2.2.2. Experimental diets

Garlic bulbs and dried wakame were bought from local stores in Sapporo, Hokkaido Japan. Propolis powder was bought from Earthship Co., Ltd. (Tsukahara, Takatsuki-shi, Osaka Japan). Experimental diets consisted of garlic, propolis, and wakame supplemented diets as GD, PD, and WD, respectively. Standard laboratory medaka diet (MD) served as the control diet. Proximate composition of control diet and percentage inclusion of test dietary sources is shown in Table 8. Inclusion levels for test dietary sources were in accordance to inclusion ranges from other studies in literature (Meurer *et al*., 2009; Dadgar *et al*., 2017; Saleh *et al*., 2015; Eirna-liza *et al*., 2016; Chesti and Chauhan, 2018; Moutinho *et al*., 2018; Hassan *et al*., 2019; Kamunde *et al*., 2019). Raw materials for the experimental diet formulation were cleaned, dried, weighed out using a scale (HF-200, A&D Co., Ltd. Tokyo, Japan), homogenized and passed through a 200 µm sieve. Material portions for each diet were then weighed out and mixed thoroughly with a digital mixer (AE300L-H, Shenzhen, China) with 0.5% water to form dough. Dough was placed on a 0.5 mm metal plate to

produce small sized pellets suitable for fish to consume. All diets were freeze dried and stored in the refrigerator at - 4°C prior to feeding.

Table 8. Proximate composition and inclusion levels of experimental diets

Proximate composition (%)	Control Diet (MD)	Garlic Diet (GD)	Propolis Diet (PD)	Wakame Diet (WD)
*Protein	>33	>33	>33	>33
*Fat	>4.0	>4.0	>4.0	>4.0
*Fiber	<2.8	<2.8	<2.8	<2.8
*Ash	<14	<14	<14	<14
*Water	<9	<9	<9	<9
Inclusion level (%)				
Control diet	100	96	96	95
Garlic	0	4	0	0
Propolis	0	0	4	0
Wakame	0	0	0	5

* As provided by feed manufacturer (Medaka fish feed granule type, DCM Brand Japan)

To verify suitability of experimental diets and inclusion rates, a 35 day feeding trial was carried out to assess dietary performance. 200 juveniles (0.21 ± 0.09 g) were randomly assigned to 3 treatment groups and 1 control in transparent fiberglass aquaria (25 liter capacity each) and fed experimental diets. Fish were fed to satiation daily (7:30am and 6:30pm) on MD or GD, PD, and WD. Uneaten feeds were removed 2 h post-feeding and fecal matter removed prior feeding and water replacement, using a suction hose. Water was replaced daily with dechlorinated tap water (up to 70%) in all groups, and water quality parameters were at optimum levels across all treatments (Table 9). Live fish were sampled

weekly in triplicate from each diet treatment group to obtain data for fish condition indices, namely weight, length, and condition factor.

2.2.3. Experimental fish and study design

All fish experiments were carried out in conformity to the rules approved by the Institutional Animal Care and Use Committee of Hokkaido University, Japan. A total of 550 (0.21 ± 0.09 g) *Oryzias latipes* juveniles were purchased from DCM, Hokkaido, Japan, and used in the study, following laboratory acclimation. Experimental design is shown in Fig. 8.

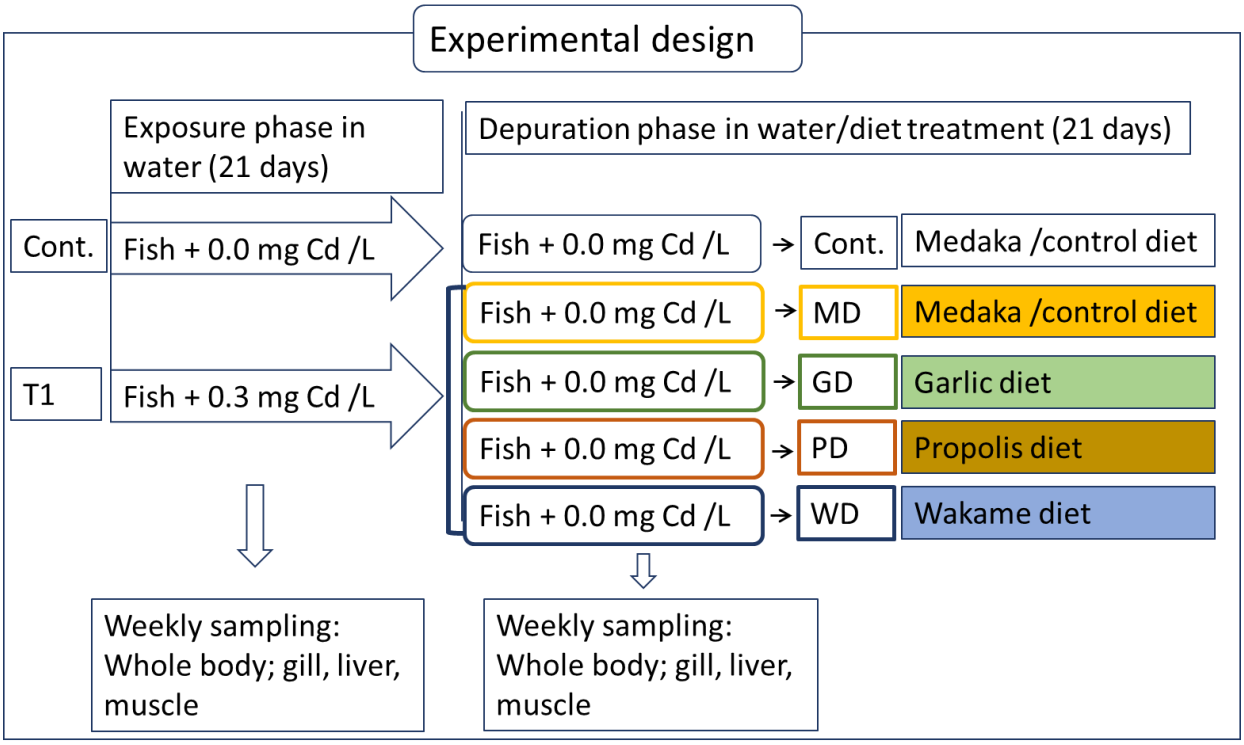

Fig. 8. Experimental design

Table 9. Water physico-chemical parameters during Cd exposure (Week 1 - 3) and depuration (Week 4 - 6) phases

	Week 1-3		Week 4 – 6				
Parameters	Cont.	T1	MD	GD	PD	WD	Reference /nominal
pH	7.57± 0.06	7.6± 0.10	7.57± 0.06	7.53± 0.06	7.5± 0.10	7.57± 0.06	6.8 - 8.5
Alkalinity (mg /L $CaCO_3$)	67.15 ± 0.15	67.25± 0.15	67.4± 0.57	67.16± 0.19	68.26± 0.53	67.51± 0.64	50 – 150
Hardness (mg /L $CaCO_3$)	88.7± 1.15	87.30± 0.84	87.03± 1.89	85.30± 1.51	87.77± 0.84	87.90± 1.15	75 – 200
DO (mg /L)	8.40± 0.10	8.50± 0.10	8.53± 0.06	8.57± 0.06	8.50± 0.10	8.53± 0.06	> 4
Temp. (^{0}C)	20 ± 0	20 ± 0	20 ± 0	20 ± 0	20 ± 0	20 ± 0	20 – 30
Total ammonia (mg /L)	nd	nd	nd	nd	nd	nd	0
Nitrite (mg /L)	nd	nd	nd	nd	nd	nd	0
Nitrate (mg /L)	3	3	2.8	2.8	2.8	3	< 200
Cd (mg /L)	nd	0.28 ± 0.01	nd	nd	nd	nd	0.3

Results = Mean ± SD (n = 6); Cont. = 0.0 mg L^{-1} Cd fed control diet; T1 = 0.3 mg L^{-1} Cd fed control diet; MD = medaka diet (depuration phase, 0.0 mg L^{-1} Cd); GD = garlic diet (depuration phase, 0.0 mg L^{-1} Cd); PD = propolis diet (depuration phase, 0.0 mg L^{-1} Cd); WD = wakame diet (depuration phase, 0.0 mg L^{-1} Cd); nd = not detected; Reference for standards: Lawrence and Mason, 2012

Cd exposure concentration for the study represented typical concentration scenarios in industrial areas, areas close to mines, or areas adjourning drainage basins and disposed untreated sludge wastes. For example, in the Pb and Zn mining communities in southeastern Nigeria, Obasi and Akudinobi (2020) reported water Cd contents in the range 0.115 – 0.509 (mg /L). Also, in Airport Lake, Dhaka, a mean value of 0.22 mg Cd L^{-1} was

reported (Mokaddes *et al*., 2013). Thus, a concentration of 0.3 mg Cd L^{-1} was selected for the study, following results from preliminary studies (Fig. 9). This concentration was considered as a representative average of reported values in literature in Cd polluted areas, and therefore environmentally relevant (Abdallah, 2008; Ohimain *et al*., 2008; Fakayode and Onianwa, 2002). The selected concentration (0.3 mg Cd L^{-1}) also indicated levels of expected tissue Cd uptake, as well as fish mortality and survival rates during Cd exposure experiment.

Cd exposure and depuration experiments were carried out in 2 phases – phase 1 (Cd exposure) and phase 2 (Cd depuration), with a total of 350 fish juveniles. In phase 1, fish were randomly assigned to 2 aquaria tanks – the control tank (25 L) and the Cd exposure tank (60 L). Fish were exposed to 0.3 mg L^{-1} Cd (T1) or 0.0 mg L^{-1} Cd (control) in water. All fish were fed control diet (MD) in entire phase 1 (Fig. 8). During phase 2, surviving fish from T1 were randomly distributed into 4 aquaria tanks (25 L each) such that total body weight in each treatment did not differ significantly ($p > 0.05$) (Fig. 8). Fish were exposed to 0.0 mg L^{-1} Cd and fed to satiation on either of GD, PD, WD, and MD diets (Fig. 8). In each phase, fish were reared for 21 days, while aquaria conditions and optimum water quality parameters were maintained as previously described. Sampling was also done according to previous procedure.

2.3. Sample collection

2.3.1. Anesthesia, sample excision, and biometrics

Samples were collected weekly during phase 1 and phase 2, respectively. Prior to sampling and anesthesia, at least 4 fishes were randomly selected from each treatment tank, fasted for 24 h in separate holding tanks with identical water quality parameters as culture aquaria tanks. This was to ensure emptying of gut and reduced fecal contamination. Water was replenished and fish were then anaesthetized with MS 222 (Tricaine methanesulfonate, ms-222) at 100 mg L^{-1}. Dosage was decided following exposure trial. Loss of equilibrium, decreased respiratory rate observed through opercula movement, and reduced reflex to external stimuli with an object indicated suitability for measurements and surgery. Tissue samples (liver, gill and muscle) were excised in triplicate, carefully and quickly under a dissecting microscope. Samples were washed with distilled water, weighed, and collected in autoclaved 2 mL-tubes and stored at -20 °C for further analysis. All equipment for surgery was adequately sterilized in advance. For biometrics measurement, live individual fish total length was measured using high precision digital Vernier caliper (Mitutoyo 536-221, Carlsbad, CA) on a fish measuring board, while weight was measured with a weighing scale (HF-200, A&D Co., Ltd. Tokyo, Japan). Upon complete recovery from effects of the anesthesia, fish were transferred from recovery holding tanks to respective aquaria.

2.3.2. Preparation of samples for acid digestion

Excised tissue samples (gill, liver, muscle) were submitted to wet digestion in the presence of concentrated nitric acid and heat to dissolve sample matrix prior Cd bioconcentration

analysis. Briefly, 1 mL of HNO_3 was added to each eppendorf tube with 0.1 to 0.2 g of samples and heated at 100°C for 1 hr. Digests were further filtered using 0.45 µm filters. Standard solutions were prepared by diluting certified stock solution Cd with ultra-pure water to desired concentration ranges. A 4 point calibration curve was used for tissue Cd bioconcentration analysis.

2.3.3. Preparation of tissue homogenates for MT analysis

For MT analysis, whole fish, and also gill, liver and muscle tissues were manually homogenized for 5 min in adequate volume (2-5 folds) of 40 mM Tris-HCl buffer, pH 7.4 under ice. Resulting homogenates were centrifuged at 8000 rpm for 30 min at 4°C with a refrigerated centrifuge (Kubota 3520, Tokyo, Japan). Supernatants from homogenates were used to measure protein concentration, and MT induction in the tissues. The quantity of protein in samples was determined spectrophotometrically (Bio-Rad; Hercules, CA, USA) using a protein assay dye reagent, the Coomassie blue reagent (Bradford, 1976).

2.3.4. Measurement of Cd concentration in water

For water Cd content, samples were collected from aquaria tanks in triplicate upon water exchange in aquaria and subjected to atomic absorption spectroscopy (AAS) analysis (Atomic Absorption Spectrophotometer, Hitachi Ltd. Tokyo Japan, 180-30). Briefly, sampling bottles were first rinsed with deionized water and HNO_3 and dried prior to collection. Samples were acidified with 2% HNO_3, boiled for 30 min on hot plate at 100°C, and filtered with 0.45 µm filters. Volumes were appropriately adjusted and made up with milli Q water before analysis. In AAS, the detection limit (µg g^{-1}) was 0.001, and

concentration was calculated from standard curve. The validity of the measurements was confirmed at the beginning and end of the experiment by measuring samples of known concentrations in the laboratory. Dissolved oxygen (mg /L) was measured with portable dissolved oxygen meter (HQ 40d, HACH Japan).Water pH was measured with portable EC pH meter (WM-32EP, TOA DKK, Japan). Water hardness (mg /L $CaCO_3$), alkalinity (mg /L $CaCO_3$), total ammonia (mg NH_3 L^{-1}), nitrite (mg /L) and nitrate (mg /L) were measured with water pack test kits (Kyoritsu chemical check lab., corp., Japan).

2.4. Sample analysis

2.4.1. Measurement of MT content

The MT content in the sample was measured by ELISA kit (Frontier-Science, Ishikari, Japan) following manufacturer's protocol. Briefly, 96-well microtiter plate was first washed with PBS solution. Next, 50 µL of standard or sample solution was added in each well, followed by addition of 50 µL first antibody in each well. The microtiter plate was then incubated at room temperature for 1 hr. Following incubation, each well of the microtiter plate was washed three times with 350 µL washing buffer, followed by addition of 100 µL second antibody. The plate was incubated for another 1 hr at room temperature, after which wells were washed as before. Then, 100 µL of substrate mixture was added to each well in a dark room. Following this, 50 µL of stop solution was added to stop the reaction. Then, absorbance of the solution was measured at 450 nm in a microplate reader, and the concentration was calculated from a standard curve of MT protein.

2.5. Data and statistical analysis

All statistical analyses were performed with IBM SPSS software, version 20.0. Data from study were subjected to one-way ANOVA. Duncan's post hoc multiple comparisons were applied to distinguish significant difference among mean values. Statistical significant difference was considered at $p < 0.05$. Results from data are presented as means ± S.D.

2.6. Results

2.6.1. Determination of appropriate Cd concentration for future study

Results on 3 weeks Cd bioconcentration and 3 weeks depuration following Cd concentration determination experiment is shown in figure 9. The purpose was to i) identify Cd bioconcentration levels at the respective exposure concentrations; ii) identify Cd persistence in fish tissues during/after depuration, which in turn necessitate dietary treatment in main study; and iii) decide what Cd concentration to use for the main study, based on results obtained. For the determination experiment, 0.3 mg Cd /L and 0.6 mg Cd /L were chosen. These concentrations were a close average to previous reports in literature on Cd polluted waters in mine areas and untreated sewage sludge. At the end of week 3, T1 and T2 groups showed significant ($p < 0.05$) Cd bioconcentration (Fig. 9a). On the other hand, during Cd depuration in clean water, T1 and T2 groups showed a significant ($p < 0.05$) reduction in Cd bioconcentration. T2 groups remained significantly ($p < 0.05$) higher

than T1 groups. However, bioconcentration remained significantly ($p < 0.05$) high in both groups at the end of depuration (Fig. 9b), suggesting the need for dietary treatment.

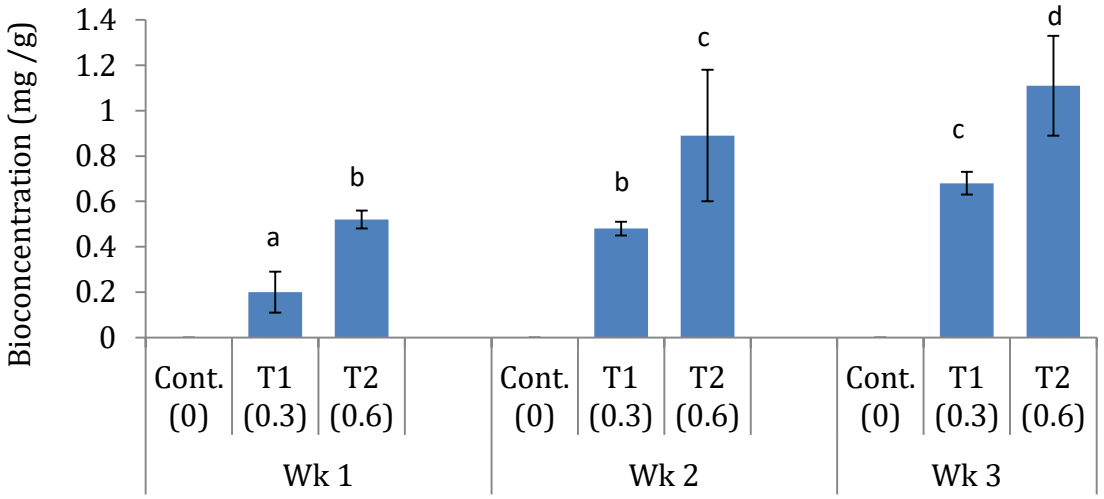

Figure 9a. Whole body Cd concentration following a 3 week Cd exposure. Figures in parenthesis indicate Cd bioconcentration in mg /L. Error bars indicate mean ± SD (n = 3). Different superscripts indicate significant differences ($p < 0.05$).

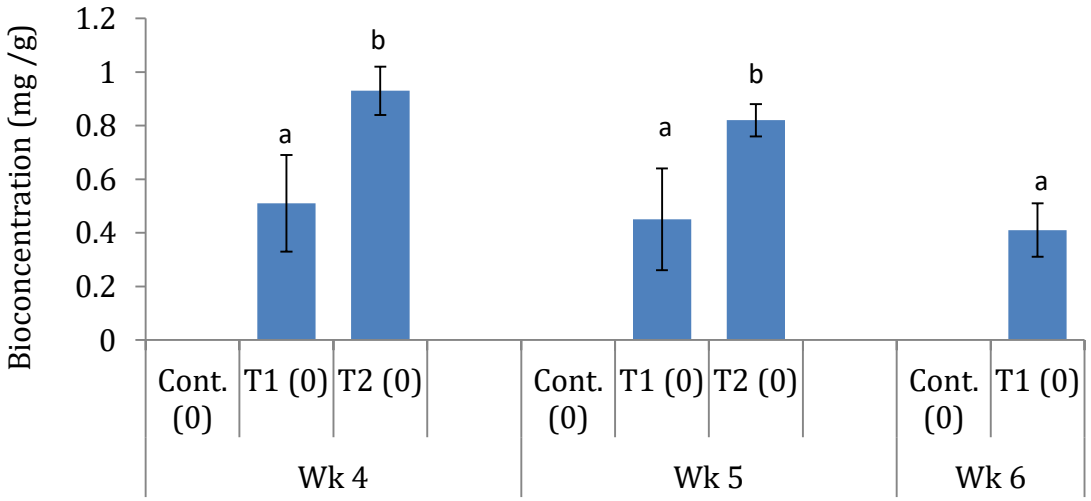

Fig. 9b. Whole body Cd concentration following a 3 week Cd depuration. Figures in parenthesis show Cd concentration in mg /L. Error bars indicate mean ± SD (n = 3). Different superscripts indicate significant differences ($p < 0.05$)

2.6.2. Fish mortality following Cd exposure and depuration experiment.

Results on Cd induced mortality during 3 weeks Cd exposure and 3 weeks Cd depuration experiments are shown in figure 10. The purpose of this experiment was to i) assess tolerance limits to Cd at the exposure concentrations; ii) estimate fish mortality in the exposure concentrations; and iii) decide exposure concentration which results in lower mortality level and higher survival. This is to ensure there is sufficient number of fish to complete the rest of the experiment during the main study. Results showed fish mortality was higher in T2 group (0.6 mg Cd L^{-1}). At the end of week 3 Cd exposure, T2 group Cd concentration exceeded the mean lethal dose (LC_{50}), while those of T1 remained lower than the LC_{50} (Fig. 10a). Cd depuration in clean water from week 4 through week 6 recorded further fish mortality which approached 100 % in T2. Thus, T1 concentration (0.3 mg Cd L^{-1}) was considered as exposure concentration for the main experiment.

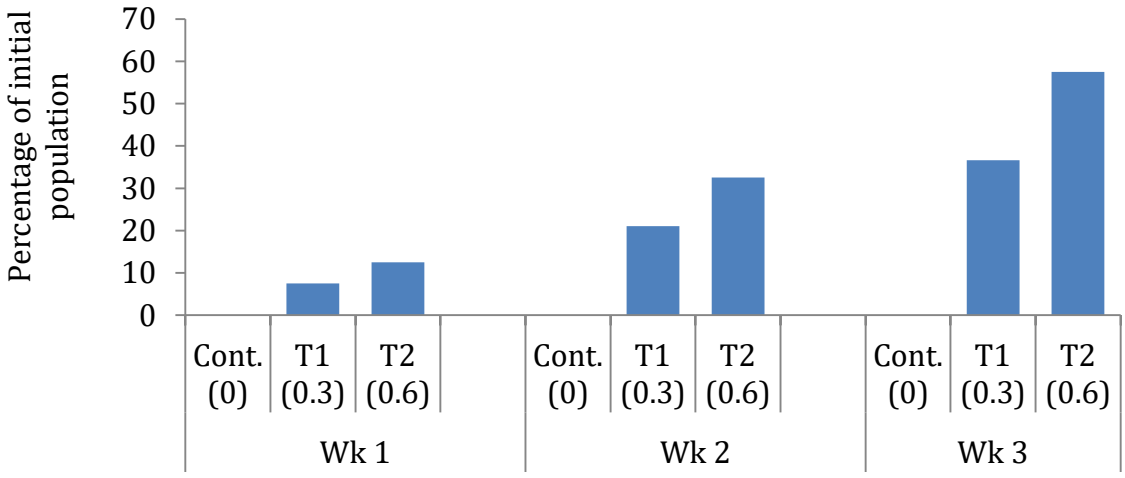

Fig. 10a. Fish mortality following Cd exposure in water. Figures in parenthesis indicate Cd concentration (mg /L) which caused fish mortality.

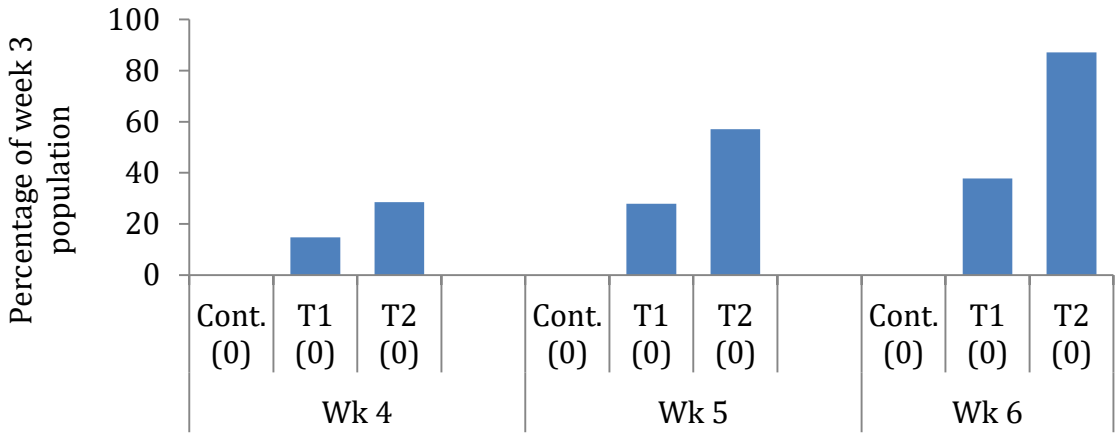

Fig. 10b. Fish mortality following Cd depuration in clean water. Figures in parenthesis indicate Cd concentration (mg /L) which caused fish mortality.

2.6.3. Dietary performance and fish condition indices

Results for diet performance trial are shown in Table 10. Weight gain in the experimental diet treatment groups GD, PD, WD compared favorably with those of MD (the control group) as they were not significantly lower ($p < 0.05$). Weight gain in GD group was significantly ($p < 0.05$) higher than those of MD, PD and WD, respectively.

Table 10. 35 days diet performance trial

	Fish biometrics			Initial		Final	
Diets	Initial weight (g)	Final weight (g)	Weight gain (g)	K_1	r^2	K_2	r^2
MD	0.106 ± 0.003[a]	0.334 ± 0.002[a]	0.228 ± 0.001[a]	0.82 ± 0.18[a]	0.94	0.96 ± 0.21[a]	0.83
GD	0.105 ± 0.002[a]	0.341 ± 0.002[b]	0.236 ± 0.000[b]	0.95 ± 0.28[a]	0.92	0.95 ± 0.28[a]	0.88
PD	0.104 ± 0.003[a]	0.333 ± 0.002[a]	0.229 ± 0.001[a]	0.98 ± 0.30[a]	0.89	1.00 ± 0.17[a]	0.89
WD	0.105 ± 0.002[a]	0.333 ± 0.004[a]	0.228 ± 0.002[a]	0.99 ± 0.26[a]	0.96	1.00 ± 0.39[a]	0.93

Results= Mean ± SD (n = 10); MD = Medaka (control) diet; GD = Garlic supplemented diet; PD = Propolis supplemented diet; WD = Wakame supplemented diet; K_1 and K_2 = Fulton's condition factor initial and final ($100*Weight/Length^3$), respectively; r^2 = coefficient of determination; Different superscripts indicate significant difference ($p < 0.05$) between control group and dietary groups.

2.6.4. Water quality parameters

AAS results showed Cd concentration in fish aquaria throughout exposure period were ± 10% of the nominal concentration (Table 9). The deviation was considered negligible, thus nominal concentration was upheld. Water physico-chemical parameters tested were within range for optimal performance of medaka fish. Therefore, effects other than those due to Cd exposure or diet treatment were justifiably checked. No significant difference ($p > 0.05$) was observed amongst treatment groups, thus eliminating any biases.

2.6.5. Tissue Cd bioconcentration and depuration kinetics

Fish tissues (gill, liver and muscle) showed a significant increase ($p < 0.05$) in Cd bioconcentration during Cd exposure phase (Fig. 11). Bioconcentration was highest in the liver, reaching about double the nominal concentration levels. During depuration, significant ($p < 0.05$) reduction in tissue Cd was recorded across all dietary treatment groups. Cd reduction ratio by diet was in the order GD > WD = PD > MD (Fig. 12).

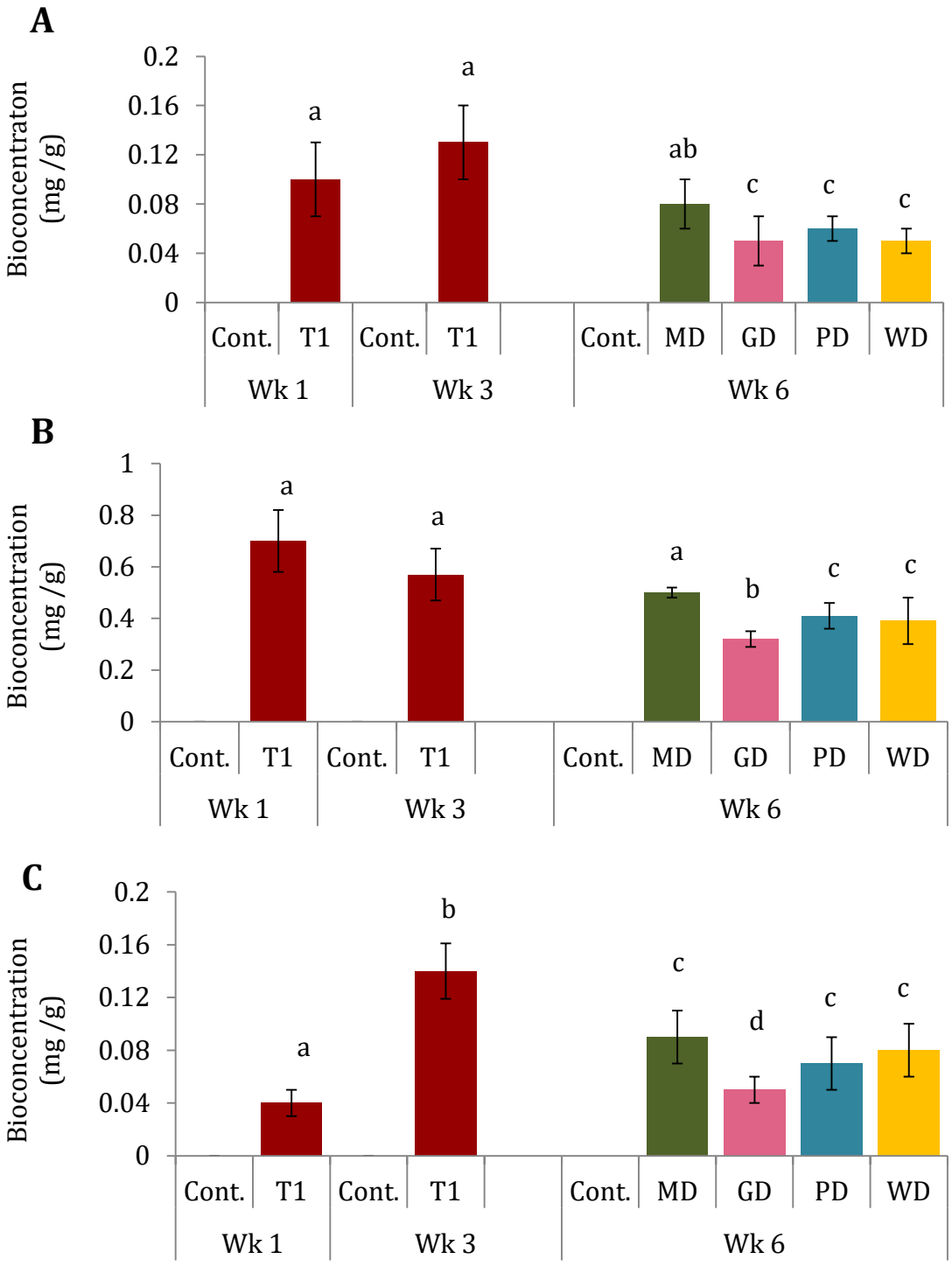

Fig. 11. Tissue bioconcentration following Cd exposure and dietary treatment. Error bars indicate mean ± SD (n = 3). A: Gill tissues; B: Liver tissues; C: Muscle tissues. Alphabet superscripts indicate significant differences ($p < 0.05$) between control groups (Cont. and MD) and treatment groups (GD, PD, WD).

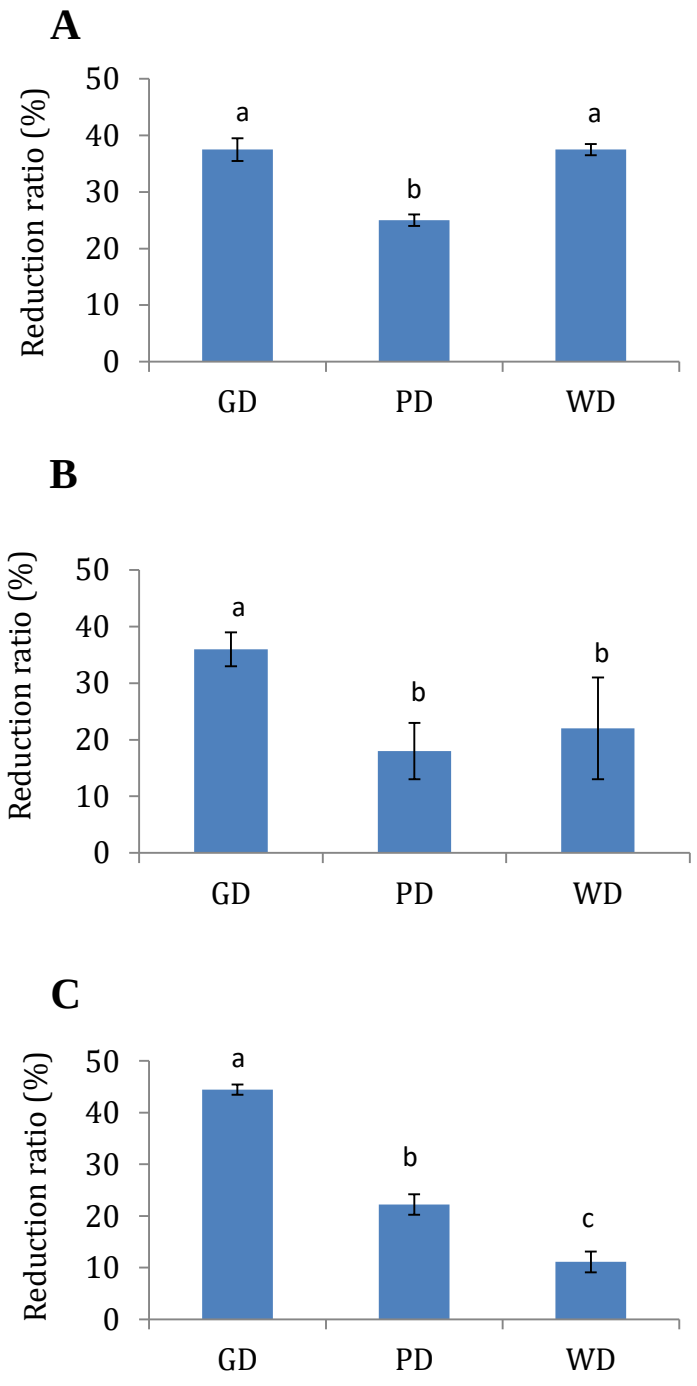

Figure 12. Cd reduction ratio by diet in A (gill), B (liver), and C (muscle) tissues. GD: Garlic diet, PD: propolis diet, WD: Wakame diet. Error bars indicate mean ± SD (n = 3). Different superscripts indicate significant differences ($p < 0.05$) between diet groups. Alphabet superscripts indicate significant differences ($p < 0.05$).

2.6.6. MT expression

Cd exposure induced a significant ($p < 0.05$) increase in MT expression across the gill, liver, and muscle tissues. Elevated MT values were sustained through Cd exposure period across all sampled tissues, except in the gill tissues where it was significantly ($p < 0.05$) reduced. Dietary treatments however, caused a significant ($p < 0.05$) increase across all sampled tissues, including in the gills where they were significantly reduced during Cd exposure in week 3 (Fig. 13). Magnitude of expression was least in gill tissues and highest in liver tissues. MT values were further correlated with Cd bioconcentration to verify the magnitude of relationship. Results showed a strong positive correlation between MT expression and Cd bioconcentration (Fig. 14).

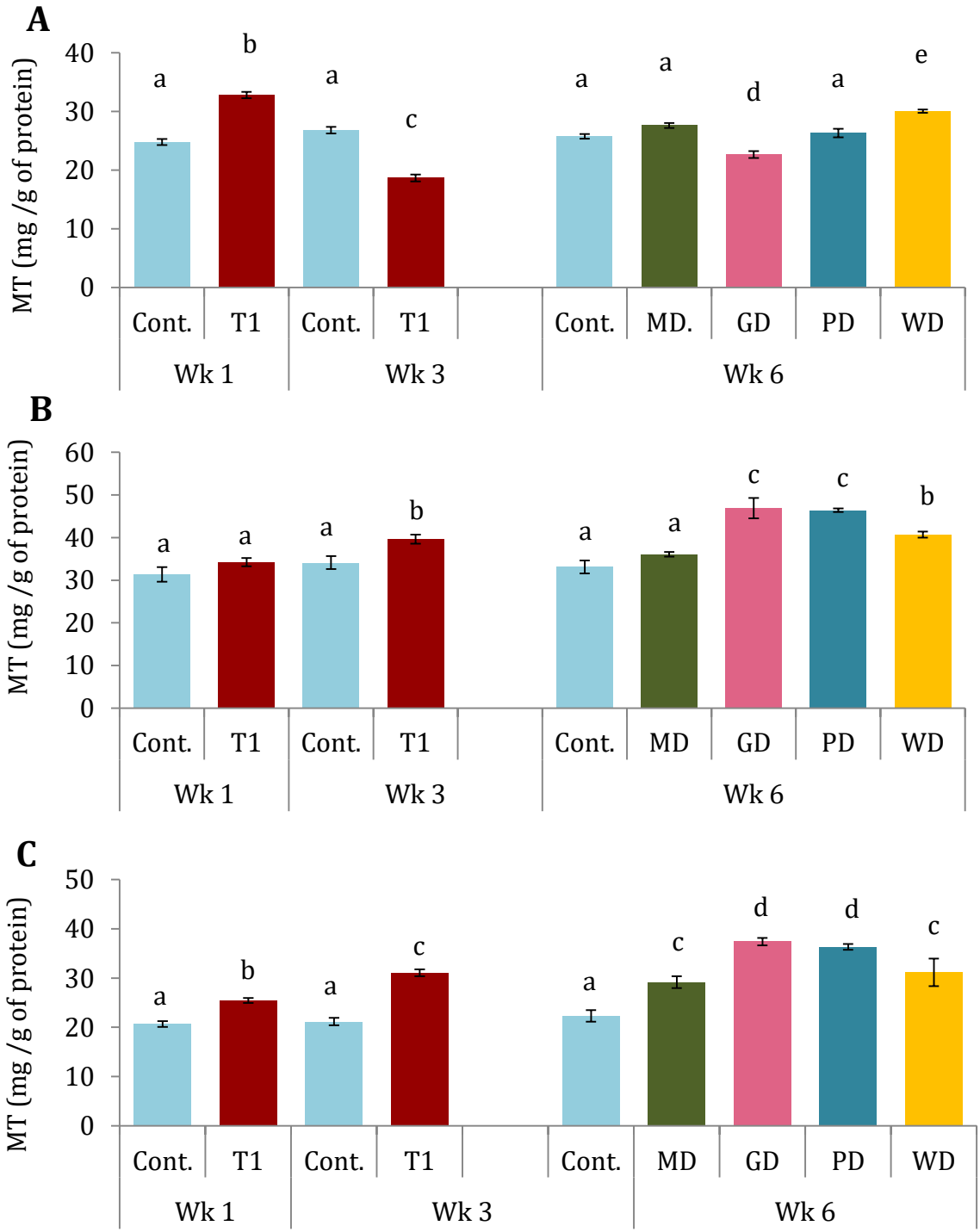

Fig. 13. MT content following Cd exposure and dietary treatment. Error bars indicate mean ± SD (n = 3). A: Gill tissues; B: Liver tissues; C: Muscle tissues. Alphabet superscripts indicate significant differences ($p < 0.05$) between control groups (Cont. and MD) and treatment groups (GD, PD, WD).

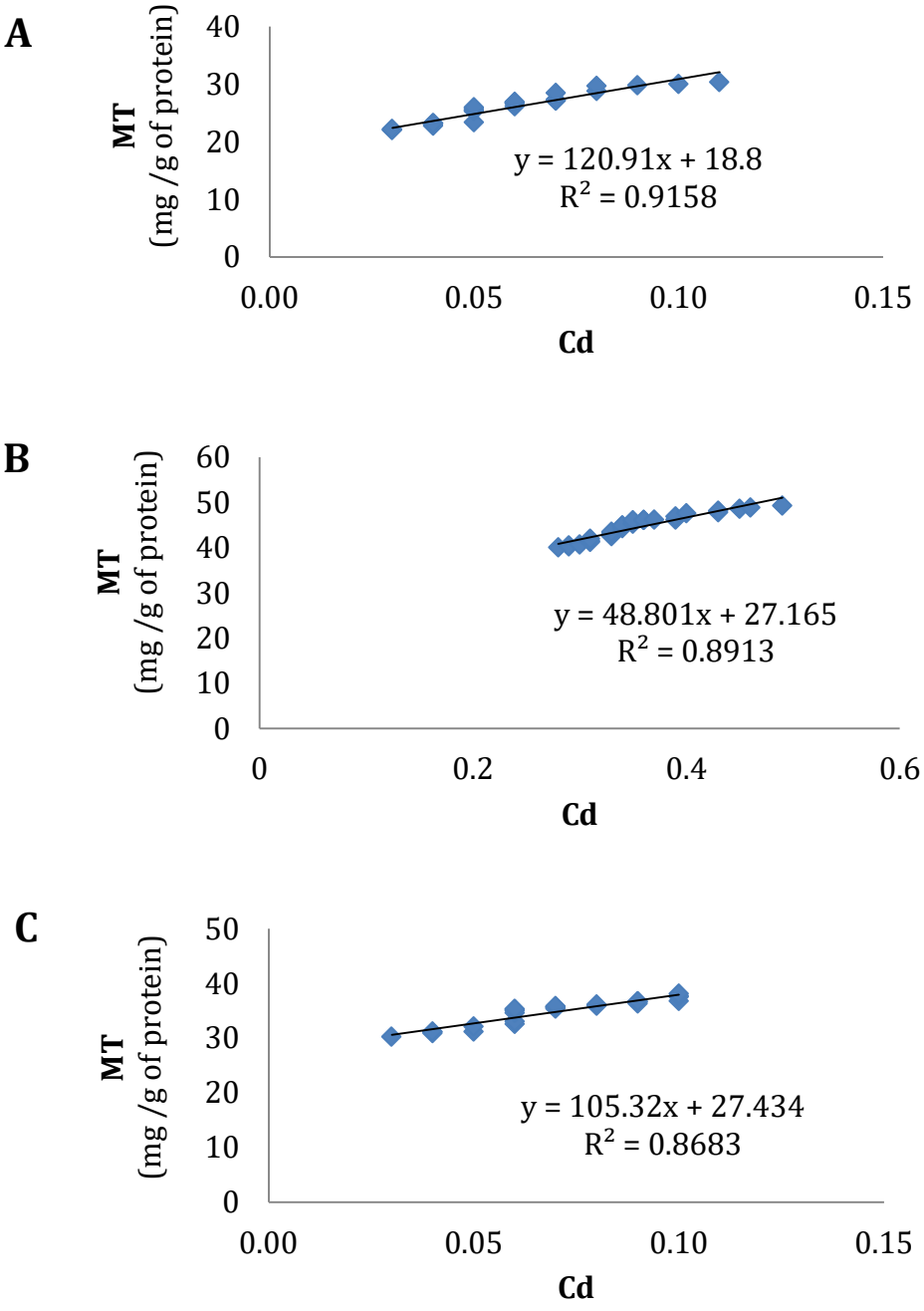

Fig. 14. Correlation analysis between MT expression and Cd bioconcentration

2.7. Discussion

The ubiquity, persistence, and highly cumulative nature of Cd pose great danger and health risk to fish and successive fish consumers along the food chain. Its toxic effects on biological tissues are well known. However, in order to exhibit toxic effects, the biologically significant ionic form of Cd, Cd^{2+}, binds to many bio-molecules, and these interactions underlie the toxicity mechanisms of Cd (Moulis, 2010). Interventions, therefore, which reduce heavy metal uptake and tissue concentration, or promote their elimination form the body, seem very logical in mitigation against Cd toxicity. Against this backdrop therefore, the prospects of dietary supplemented garlic, propolis, and wakame to reduce Cd uptake and enhance their elimination were assessed. Findings from this study corroborate those reported in previous studies in rats and other experimental models, fed similar experimental diets (Tandon *et al*., 2001; Nada and Azab, 2005).

In order to verify these findings, it was necessary to first ensure all biases were eliminated, and that observed effects were due to those of Cd exposure, beside dietary treatments. To achieve this, optimum water quality conditions for Japanese medaka were maintained for all treatment groups. It is noted that fish have species-specific and sometimes even life stage–specific optimal ranges for each water quality parameter. When parameters fall outside the acceptable range, fish become stressed and more susceptible to disease (National research Council, 2011). Results showed water quality parameters were within optimum range for the species and did not vary among treatments throughout the study period.

In this study, fish were exposed to a nominal concentration of 0.3 mg L^{-1} Cd. This concentration which was environmentally relevant was verified through preliminary experiments. The accumulation of Cd in the liver and other tissues of exposed fish has been reported by numerous studies (Abdeltawwab *et al*., 2010; Giari *et al*., 2007; Xiong *et al*., 2020). In this study, Cd bioconcentration was highest in the liver tissues (about double the nominal exposure concentration) and least in the muscle tissues. This finding may be due to the respective roles of the tissues with respect to xenobiotics metabolism or homeostasis. For example, fish liver is a vital organ concerned with basic metabolism and is the major organ of accumulation, biotransformation and excretion of contaminants in fish (Figuiredo-Fernandes *et al*., 2006). Fish gills are multifunctional organs involved in ion transport, gas exchange, acid–base regulation and waste excretion (Dang *et al*., 2001). They account for about 50% of the surface area of a fish, thus constitute a major target organ for waterborne xenobiotics (Playle, 1998). Moreover, similar finding was reported in Cd exposed rare minnow (Xiong *et al*., 2020). Low Cd concentrations in muscle tissues were also reported (Kim *et al*., 2004). Results from this study showed greater tissue Cd depuration in GD, PD, and WD, relative to MD (the control diet group). This finding supports the ameliorating potentials of the supplemented dietary sources garlic, propolis, and wakame, respectively. Garlic contains many organosulfur compounds, OSCs which have reputable pharmacological and antioxidative properties, like allicin and diallyl disulfide (DADs) (Boonpeng *et al*., 2014; Kay *et al*., 2010; Amagase, 2006; Robert *et al*., 2001; Jose *et al*., 2003; Lawal and Elizabeth, 2011). The complexation of Cd (soft Lewis acid) with free “lone pair” of electrons on sulfur atom (soft Lewis base) of various

organosulfides present in garlic and the subsequent excretion of such complexes (Tandon *et al*., 2001) may also be a plausible reason. Propolis, a natural product of honey bees from gums of various plants contains more than 300 chemical compounds, including flavonoids, amino acids and fatty acids (Bankova *et al*., 1995). The physiological properties of these compounds such as antiviral, antibacterial, anticancer, and antioxidative properties have been reported (Ahn *et al*., 2009; Jung *et al*., 2008; Kujumgiev *et al*., 1999; Watabe *et al*., 2004). Wakame on the other hand has been reported to contain numerous phytochemicals and biologically active compounds such as proteins, polysaccharides fucoxanthin, macro and trace elements, as well as physiologically important fatty acids (Zhang *et al*., 2014). Sato *et al*. (2002) further reported wakame can regulate physiological disorders. *In vitro* studies of sulfated polysaccharide fractions from wakame exhibited scavenging abilities on superoxide radicals, hydroxyl radicals, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and metal chelating (Hu *et al*., 2010).

Depurative interventions of experimental diets on Cd uptake and bioconcentration in fish were expressed by modulations in metallothionein levels upon exposure. Metallothioneins are part of the inherent homeostatic mechanisms in fish which effects regulated storage (uptake) and detoxification (Wood, 2012). Their cysteine-rich thiol groups enable their high-affinity of binding to Cd. In this study, Cd exposure increased MT level in all studied tissues through week 3, except in gill tissues where it was significantly reduced in the last week of exposure. Interestingly, their order of restoration during depuration was in the order GD > PD = WD > MD, except in the gills where it was in the order GD < PD < MD < WD. It is not clear the reason for the discrepancy, but Carginale *et al*. (1998) reported Cd-

induced differential MT isoform accumulation in the Antarctic ice fish. The authors noted that endogenous transcripts consisted mostly of MT-II, whereas the MT-I transcript was preferentially accumulated only in response to the cadmium salt. Also, the strong positive correlation between MT expression and Cd bioconcentration further shows that MT expression was actually induced due to Cd uptake in tissues. The Cd binding activity of MT and subsequent reduction of Cd within tissues during dietary treatment is further implied.

2.8. Conclusion

Results from this study indicate the supportive roles of the supplemented diets during depurative interventions in Cd exposed subjects, as opposed to their absence. Overall, the magnitude of recuperation in juvenile medaka fish fed experimental diets was more significant compared with those in MD (the control diet group). This manifested in significant reduction in Cd bioconcentration, and significant upregulation in MT expression across diets. Generally, dietary supplementation significantly increased recuperation by promoting Cd elimination and reducing tissue bioconcentration, thereby lowering chances of biomagnification and resultant health risks. Inclusion of these dietary sources in fish feed indicates great promise in aquaculture settings in areas where Cd pollution and toxicity may raise fish health and safety concerns.

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Chapter 3: Comparative assessment of cadmium induced toxicity and recuperation in Japanese medaka under three dietary regimes

Abstract

Cadmium (Cd) induced oxidative stress and harmful effects can be protracted during recuperation in fish. This can potentially overcome protection by endogenous antioxidants and affect fish recuperation. Thus, this study assessed the antioxidative and ameliorating support of dietary supplemented garlic (GD), propolis (PD), and wakame (WD) on Cd exposed Japanese medaka. In a 21-day exposure, control (0.0 mg Cd /L in water – Cont.) or Cd-treatment (0.3 mg Cd /L in water – T1) fish groups were fed medaka diets. Surviving fish in T1 were further depurated for 21 –days and fed GD, PD, WD, or medaka diet (MD). Gill, liver, and muscle tissues were assessed weekly for the antioxidants superoxide dismutase (SOD) and total glutathione (GSH), and also lipid peroxidation (LPO). Results showed reduced antioxidant activity by significantly increasing LPO and significantly reducing SOD activity and GSH contents in gill and muscle tissues upon Cd exposure. In contrast, GD, PD, and WD diets significantly reduced LPO, while significantly increasing contents of GSH and SOD activity. In addition, fish mortality was reduced, and survival

rates improved. Condition indices in GD, PD, and WD groups were also significantly higher than those in MD groups. In conclusion, dietary supplementation significantly increased recuperation and tissue functions in fish, in the order GD > PD > WD > MD.

3.1. Introduction

Cadmium (Cd), like other heavy metals, are well-known environmental pollutants due to their toxicity, persistence in the environment, and bioaccumulative nature (Ali *et al*., 2019). Concern over Cd^{2+} is due to its extremely high toxicity, a long half-life in humans and being a causative agent for many diseases and disorders upon acute or chronic exposure (Järup and Åkesson, 2009; Jiang *et al*., 2014). In fish, exposure is associated with a variety of ailments, including inducement of structural and functional changes in gill, intestine, liver and kidney, alteration of antioxidant defense system and production of free radicals (Kumar and Singh, 2010). With depleted supplies of endogenous antioxidants, natural recovery from exposure and toxic effects become impeded. From this hindsight, some studies have assessed the prospects of exogenous supply of antioxidants, or of boosting their endogenous supplies through dietary and nutritional interventions. In this quest, the antioxidant potentials of plants, derived products and their bioactive compounds have been largely explored. Garlic, propolis and wakame were selected in this study based on their immune stimulating and antioxidative merits. For example, garlic offered protective effects against Cd induced toxicity in sea bass head, liver and kidney tissues (Mosbaha *et al*., 2017). Garlic also decreased the accumulation and toxic effects of lead (Pb) and Cd in the

blood, liver and kidney of male albino rats (Tandon *et al*., 2001). Kamiya *et al*. (2012) demonstrated that propolis suppressed $CdCl_2$ induced cytotoxicity of kidney tubule COS7 cells. Co-administration of propolis with Pb inhibited Pb-induced neurological toxicity in Swiss albino rats (El-Masry *et al*., 2011). Arsenic exposed *Cyprinus carpio* showed improved antioxidant effects with propolis (Talas *et al*., 2014). Fucoxanthin, the major active component in the popular seaweed, wakame (*Undaria pinnatifida*), was reported to appreciably decrease oxidative stress and apoptosis induced by $CdCl_2$ in mice (Yang *et al*., 2020). In PC12 cells, *Undaria pinnatifida* also showed radical scavenging and antioxidant activities which contributed to protective effects against iron-induced toxicity (Nishibori *et al*., 2012). As yet, studies in fish which bother on the amelioration of bioconcentration and toxicity effects of Cd are limited. Rapid recovery of a fish species from heavy metal exposure and effects will ensure its improved performance and species viability. More so, health risks to fish consumers along successive links in the food chain will be substantially reduced. Thus, this study investigated the Cd toxicity ameliorating potentials of garlic, propolis, and wakame in the supplemented diets and their respective roles in enhancing recovery in Cd exposed fish.

3.2. Materials and methods

3.2.1. Materials

Bio-Rad protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories Inc. USA (Bio-Rad, Hercules, CA, USA). $CdCl_2$. $2.5H_2O$, Cd standard solution and nitric

acid were bought from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan. Tricaine methanesulfonate, MS-222 was bought from San Diego, CA 92126, USA. SOD assay kit and GSSG/GSH quantification kits were purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Other chemical reagents used in analysis were of analytical grade.

3.2.2. Experimental diets

Experimental diets consisted of garlic, propolis, and wakame supplemented diets as GD, PD, and WD, respectively. Standard medaka diet served as the control (MD). Proximate composition of control diet and percentage inclusion of test substances is shown in Table 8.

3.2.3. Experimental fish and study design

Experimental design is shown in Fig. 15. All fish experiments were carried out in conformity to the rules approved by the Institutional Animal Care and Use Committee of Hokkaido University, Japan. A total of 550 (0.21 ± 0.09 g) *Oryzias latipes* laboratory bred juveniles were used in the study, following laboratory acclimation. Cadmium exposure and dietary treatment experiments were carried out in similar manner as reported in the previous chapter.

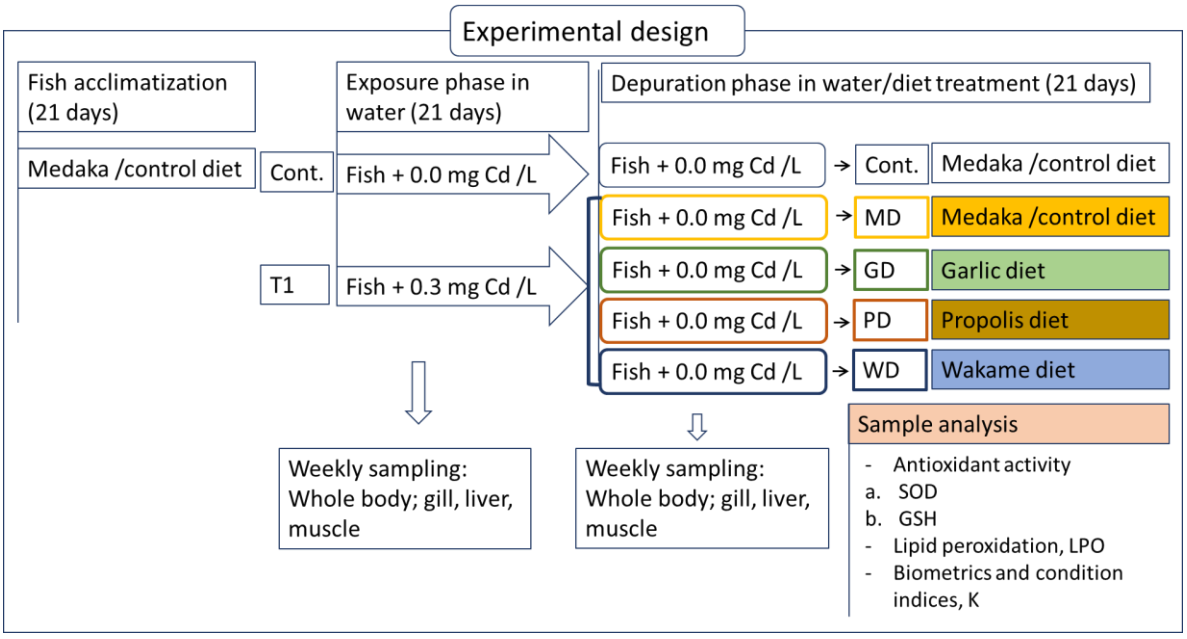

Fig. 15. Experimental design

3.3. Sample collection

3.3.1. Anesthesia, sample excision, and biometrics

Procedures for anesthesia, sample excision, and biometrics assessment were also done in line with section 2.3.1 in chapter 2.

3.3.2. Preparation of tissue homogenates for antioxidant and LPO analysis

To determine anti-oxidant enzyme and substances in the tissues, gill, liver and muscle were manually homogenized for 5 min in adequate volume (2-5 folds) of 40 mM Tris-HCl buffer, pH 7.4 under ice. Resulting homogenates were centrifuged at 8000 rpm for 30 min at 4°C with a refrigerated centrifuge (Kubota 3520, Tokyo, Japan). Supernatants from homogenates were used to measure protein concentration, and contents or activity of the

antioxidants; SOD, GSH and MT, and LPO to assess oxidative damage in the tissues. The quantity of protein in samples was determined spectrophotometrically (Bio-Rad; Hercules, CA, USA) using a protein assay dye reagent, the Coomasie blue reagent (Bradford, 1976).

3.4. Sample analysis

3.4.1. Measurement of SOD activity

Supernatants were assayed for SOD activity using SOD Assay Kit (Dojindo Molecular Technologies, Inc. Kumamoto, Japan.) following manufacturer's protocol. Briefly, 20 µL of sample solution was added to each sample and blank well in the 96 well plate provided, while 20 µL of milli-Q water was added to each blank 1 and blank 3 well. Two hundred µL of WST working solution was added to each well and mixed. 20 µL of dilution buffer was further added to each blank 2 and blank 3 well. This was followed by addition of 20 µL enzyme working solution to each sample and blank 1 well, and then thoroughly mixed. Plates were then incubated at 37°C for 20 min. Absorbance was measured at 450 nm using microplate reader (Bio-Rad; Hercules, CA, USA). SOD activity (inhibition rate %) was calculated using the equation:

SOD activity (inhibition rate %) =

$$[(A_{blank\ 1} - A_{blank\ 3}) - (A_{sample} - A_{blank\ 2})] / (A_{blank\ 1} - A_{blank\ 3}) \times 100$$

3.4.2. Measurement of GSH content

Supernatants were assayed for GSH content by the DTNB method. This involved the oxidation of GSH by the sulfhydryl reagent 2.5 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5'-thio-2-nitrobenzoic acid (TNB). Absorbance was measured at 412 nm, using a DU-65 spectrophotometer (Beckman, CA, USA). Molecular Extinction Coefficient of liberated TNB was taken as 13600 M^{-1} (Ellman, 1959) and was used to calculate GSH content in samples.

$$\frac{Sample\ absorbance - Blank\ Absorbance}{0.0136} = \text{X } \mu\text{M}.$$

3.4.3. Measurement of LPO levels

The reactive carbonyl compound malondialdehyde (MDA) is one of the major compounds formed during lipid peroxidation. The reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions forms a MDA-TBA adduct, which is measured color metrically at 530-540 nm. Assay was performed by the TBARS (TCA Method) Assay Kit (Caymen Chemical, Ann Arbour, MI) following manufacturer's protocol. Briefly, 100 µL of sample or standard was added to appropriately labeled vial. One hundred µL of TCA Assay Reagent (10%) was added to vial and swirled to mix. Then, 800 µL of provided color reagent was added to each vial and vortexed. Vials were capped and heated in an upright position in vigorously boiling water for about one hour. After one hour, vials were immediately removed and placed in ice bath to stop reaction, with further 10 min incubation on ice. Vials were then centrifuged for 10 min at 1,600 x g at 4°C. Two hundred

µL of supernatants were carefully removed from each vial in duplicate and transferred to clear 96 well plate, and absorbance read at 540 nm. MDA values for each sample were calculated from standard curve using the equation:

MDA (µM) = [(Corrected absorbance) – (y – intercept) /Slope]

3.5. Data and statistical analysis

All statistical analyses were performed with IBM SPSS software, version 20.0. Data from study were subjected to one-way ANOVA. Duncan's post hoc multiple comparisons were applied to distinguish significant difference among mean values. Statistical significant difference was considered at $p < 0.05$. Results from data are presented as means ± S.D.

3.6. Results

3.6.1. Cd exposure and fish mortality

Total fish mortality at end of Cd exposure phase was 35% in T1 (0.3 mg Cd L^{-1}) group and 0% in Cont. group (0.0 mg Cd L^{-1}). Percentage survival at the end of depuration phase was 100%, 75%, 95%, 92% and 89% in Cont., MD, GD, PD, and WD groups, respectively (Fig. 16).

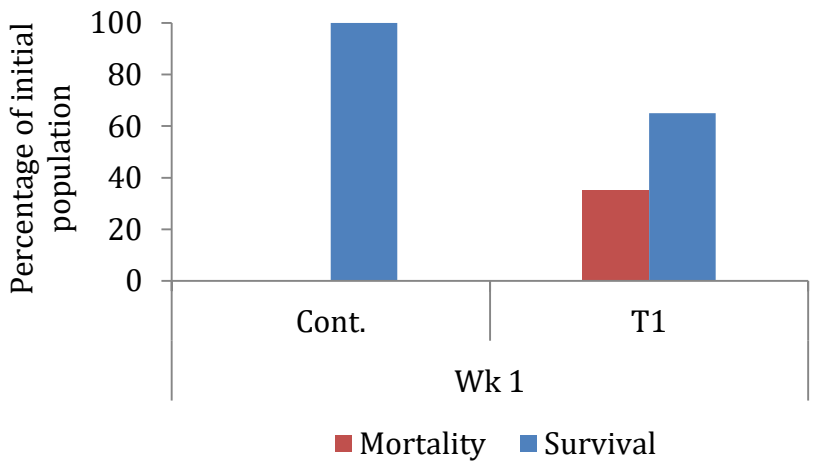

Fig. 16 a. Fish mortality and survival during Cd exposure phase

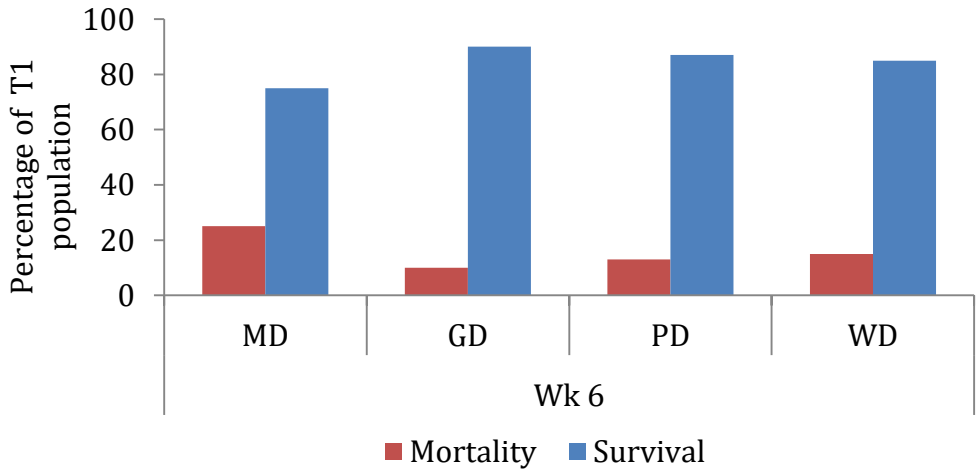

Fig. 16 b. Fish mortality and survival following dietary treatment

3.6.2. Fish biometrics and condition indices

Results for fish biometrics and condition indices are shown in tables 11 and 12. At the end of Cd exposure phase, T1 showed a significant decrease ($p< 0.05$) in fish mean body weight gain and condition factor (K), relative to those of Cont. (Table 8). On the other hand, during the depuration phase, there was significant increase ($p< 0.05$) in weight gain in GD, PD, and WD, and condition factor did not differ significantly ($p> 0.05$) between GD, PD, WD and control groups (Table 9).

Table 11. Fish biometrics and condition indices during Cd exposure (week 1 – 3)

	Cont.			T1		
	W (g)	K	r^2	W (g)	K	r^2
Initial (g)	0.193 ± 0.001	1.33 ± 0.20	0.8956	0.193 ± 0.002	1.45 ± 0.24	0.8645
Final (g)	0.207 ± 0.003	0.93 ± 0.09	0.9275	0.202 ± 0.004	1.18 ± 0.23	0.8863
Gain (g)	0.014 ± 0.002	-	-	0.009 ± 0.002	-	-

Results = Mean ± SD (n = 3); Cont. = control group (0.0 mg L^{-1} Cd); T1 = Cd exposed group (0.3 mg L^{-1} Cd); W= fish weight; K = Fulton's condition factor (100*Weight/Length^3); r^2 =coefficient of determination

Table 12. Fish biometrics and condition indices during Cd depuration and dietary treatment

Treatments	Parameters		
	W (g)	K	r^2
Cont.	0.329 ± 0.003	0.90 ± 0.13	0.8348
MD	0.323 ± 0.005	0.82 ± 0.04	0.7967
GD	0.323 ± 0.003	0.90 ± 0.14	0.9190
PD	0.318 ± 0.002	0.90 ± 0.15	0.7147
WD	0.316 ± 0.002	0.89 ± 0.16	0.8518

Results = Mean ± SD (n = 3); Cont. = control diet group with 0.0 mg L^{-1} Cd; MD = Cd exposed treatment group fed control diet as positive control; GD = garlic diet group; PD = propolis diet group; WD = wakame diet group; W= fish weight; K = Fulton's condition factor (100*Weight/Length^3); r^2 =coefficient of determination

3.6.3. SOD activity

SOD is an important, indispensable first line defense antioxidant (Ighodaro and Akinloye, 2018). In this study, SOD activity in muscle and gill tissues increased significantly ($p < 0.05$) following Cd exposure (Fig. 17). However, exposure up to week 3 caused significant decline in SOD activity in liver and gill tissues. Dietary treatment on the other hand significantly ($p < 0.05$) restored SOD activities across all treatment groups. Magnitude of restoration was least in muscle tissues.

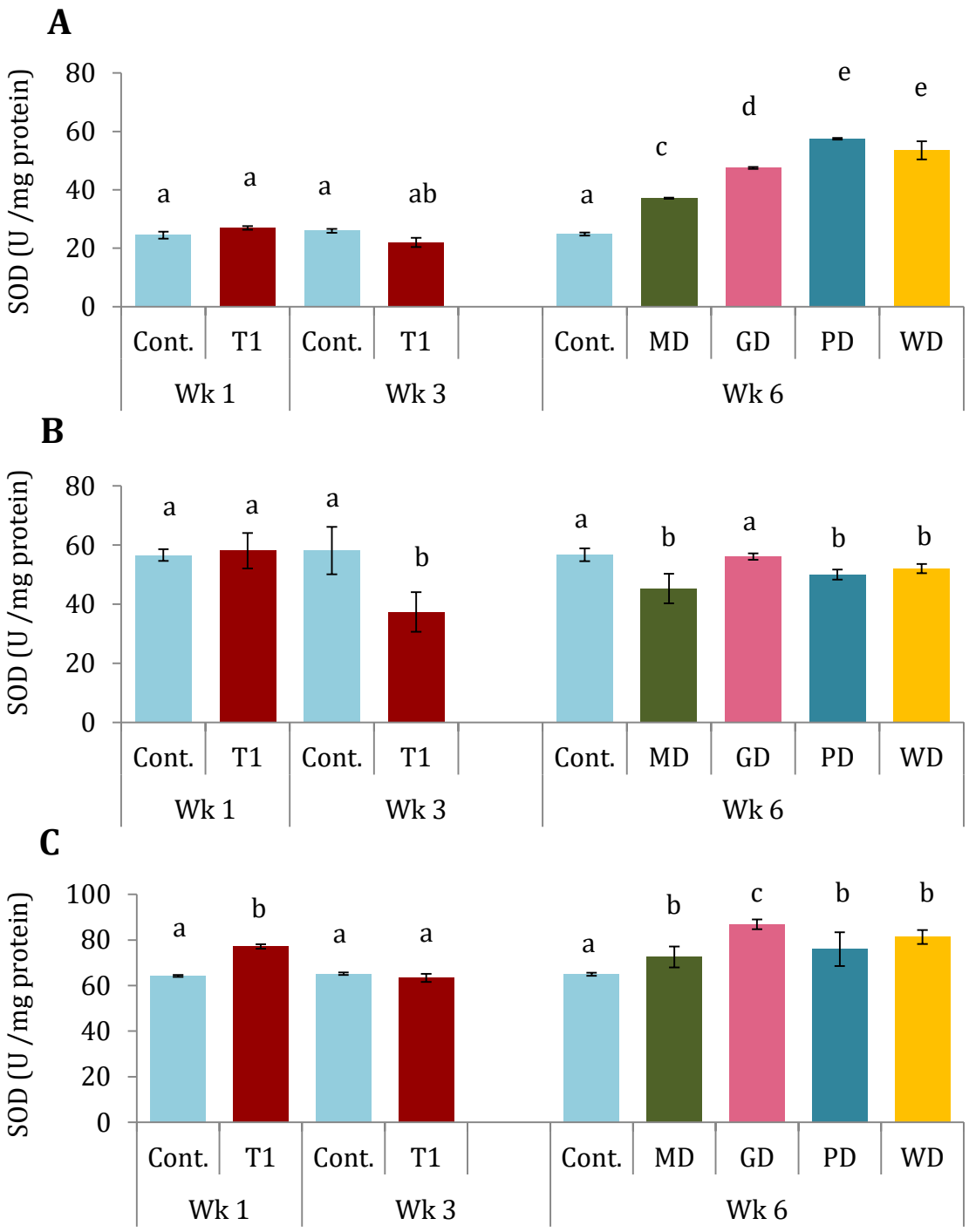

Fig. 17. SOD activity following Cd exposure and dietary treatment. Error bars indicate mean ± SD (n = 3). A: Gill tissues; B: Liver tissues; C: Muscle tissues. Alphabet superscripts indicate significant differences ($p < 0.05$) between control groups (Cont. and MD) and treatment groups (GD, PD, WD).

3.6.4. Total GSH content

GSH is a low-molecular weight, major non-protein cellular thiol, present in both eukaryotic as well as prokaryotic cells (Pavarino *et al*., 2013; Pizzorno and Katzinger, 2012). It directly acts a scavenger of oxyradical and also as an antioxidant enzyme substrate (Mozsar *et al*., 2015). Results showed a significant ($p < 0.05$) decrease in GSH content in the gill and muscle tissues, while those of the liver increased significantly ($p < 0.05$). Dietary treatment however, significantly ($p < 0.05$) restored declined GSH content across all tissues (Fig18).

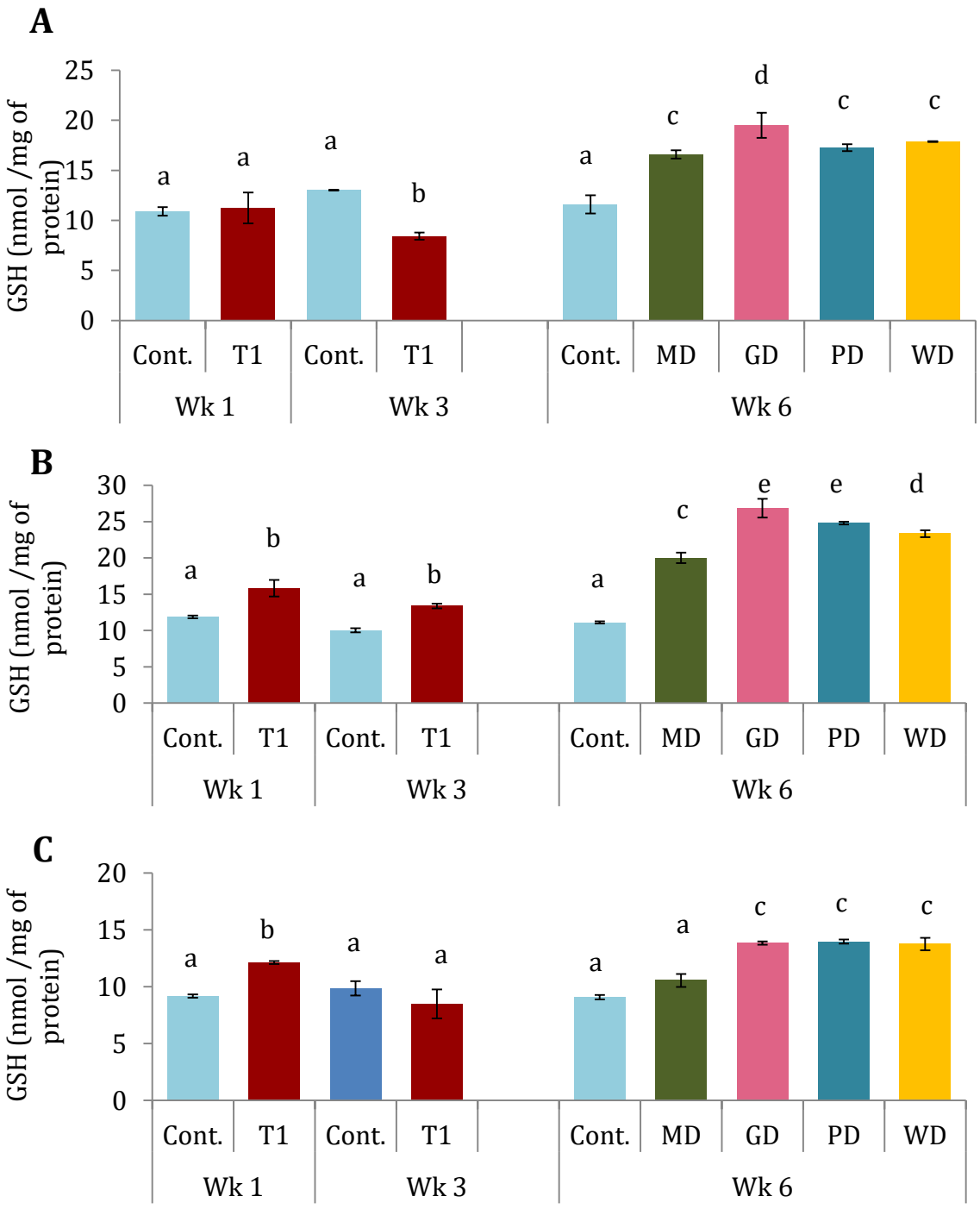

Fig. 18. GSH content following Cd exposure and dietary treatment. Error bars indicate mean ± SD (n = 3). A: Gill tissues; B: Liver tissues; C: Muscle tissues. Alphabet superscripts indicate significant differences ($p < 0.05$) between control groups (Cont. and MD) and treatment groups (GD, PD, WD).

3.6.5. Relative antioxidant ability, RAA

Correlation analysis was carried out between the enzyme superoxide dismutase SOD and the antioxidant GSH to show the relative antioxidant ability, RAA within studied tissues (Fig. 19). Results showed a weak positive correlation between SOD and GSH in gill and muscle tissues. Conversely, the liver tissues showed a strong positive correlation between SOD and GSH.

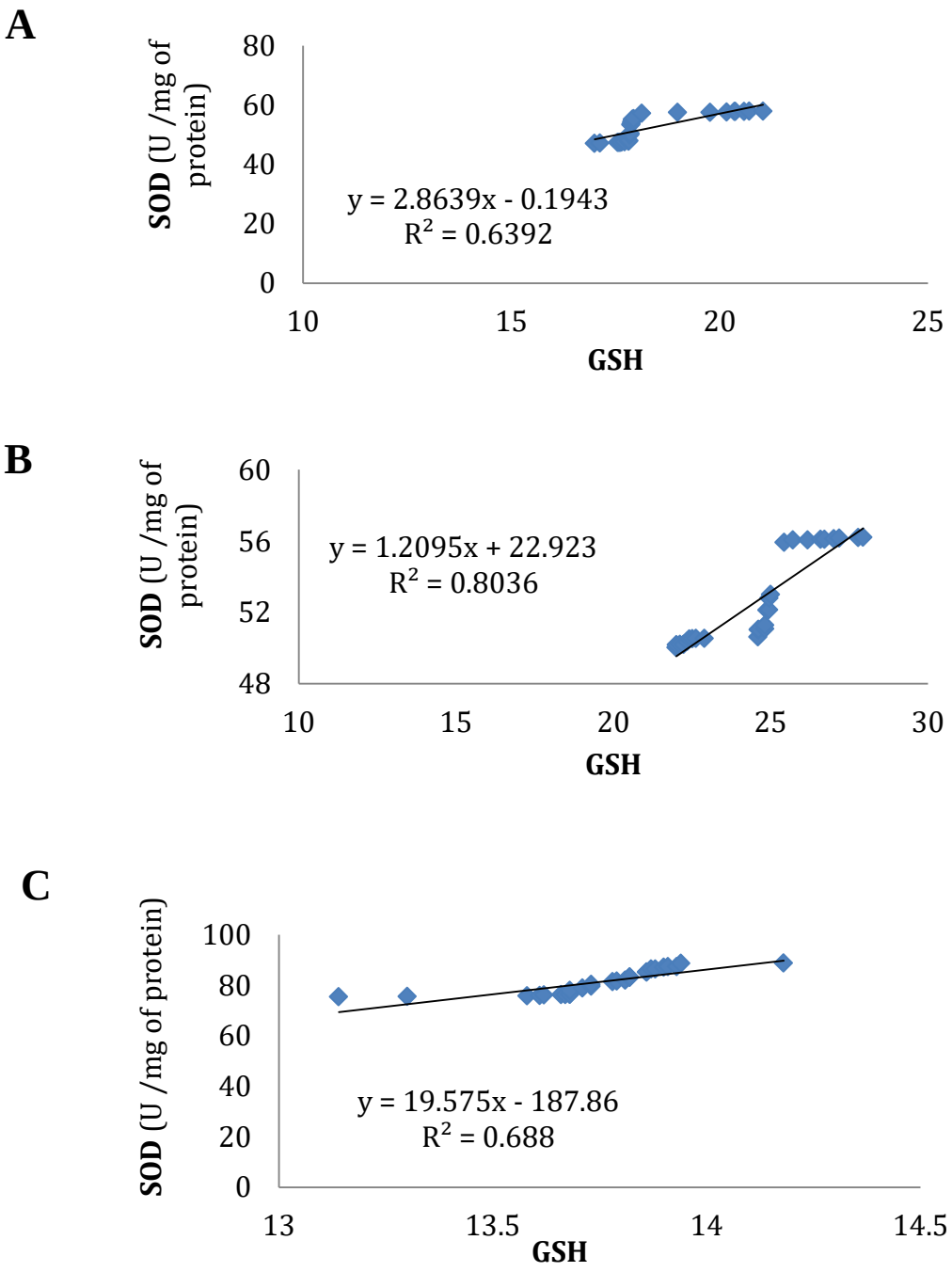

Figure 19. Correlation analysis between antioxidant enzyme, SOD and antioxidant GSH in A (gill), B (liver), and C (muscle) tissues.

3.6.6. Induction of LPO

As a proxy to measure LPO, the reactive aldehyde, MDA was measured in tissues of Cd exposed subjects. MDA is an end product of the oxidative degeneration of lipids, an indicator of the toxic effects of ROS and free radicals on cells and tissues. Following Cd exposure, results showed a significant ($p < 0.05$) elevation in tissue MDA levels. MDA levels were significantly reduced ($p < 0.05$) following dietary treatment during in the order GD < PD < WD < MD (Fig. 20).

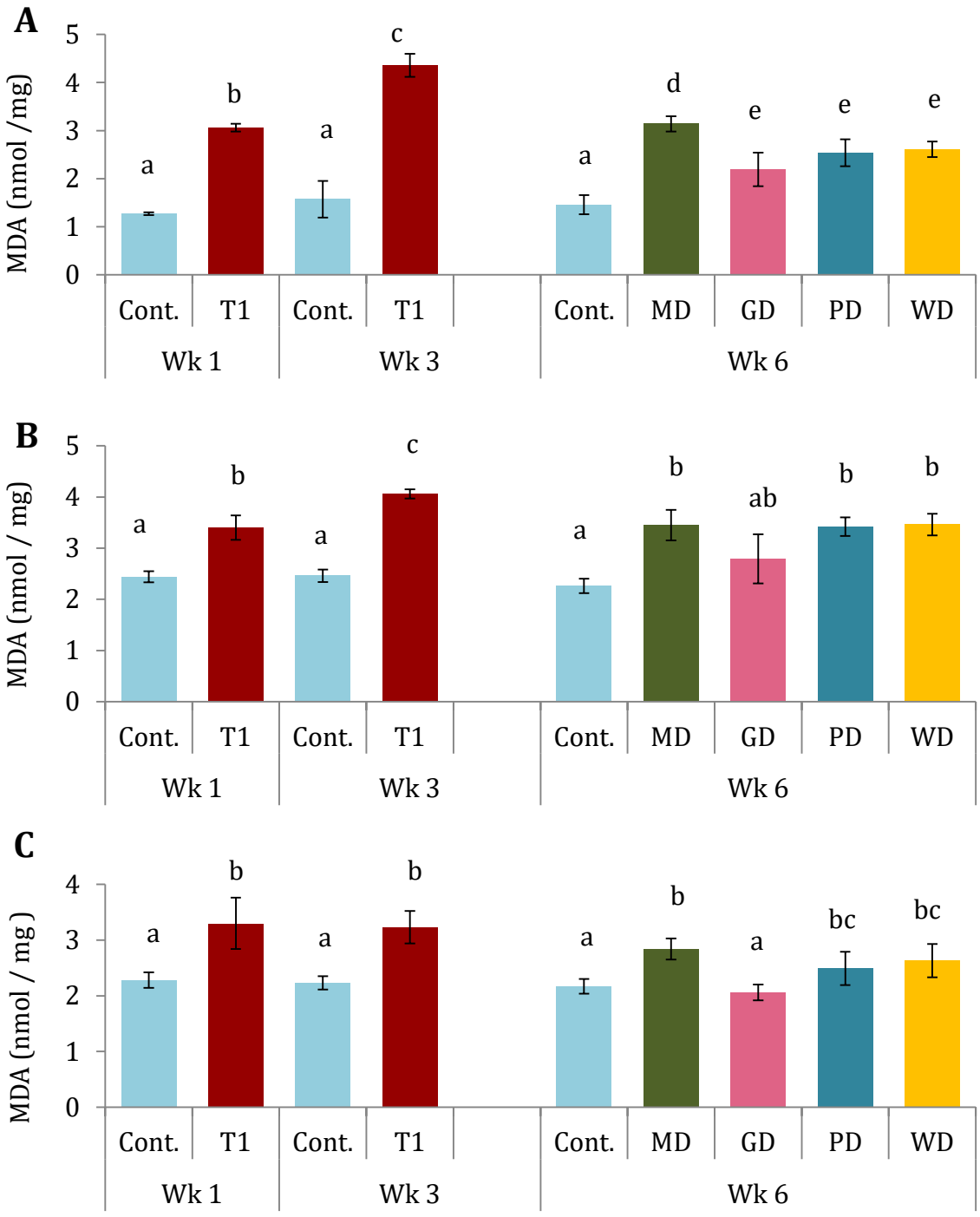

Fig. 20. LPO following Cd exposure and dietary treatment. Error bars indicate mean ± SD (n = 3). A: Gill tissues; B: Liver tissues; C: Muscle tissues. Alphabet superscripts indicate significant differences ($p < 0.05$) between control groups (Cont. and MD) and treatment groups (GD, PD, WD).

3.6.7. In diet antioxidant ability (IAA)

As a proxy to compare the dietary antioxidant ability in ameliorating Cd toxicity, the antioxidant GSH was correlated with the toxicity marker MDA. Results showed Cd toxicity reduction in the order GD > PD = WD (Fig. 21).

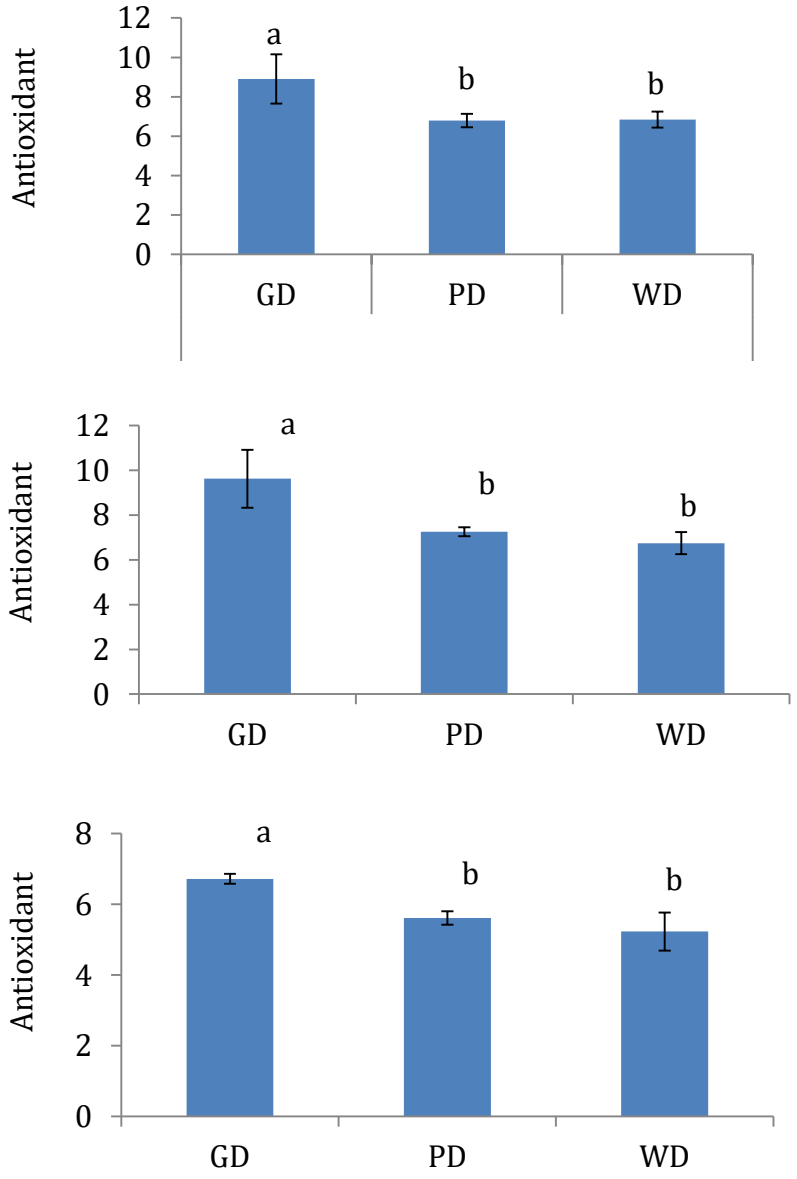

Fig. 21. In diet antioxidant ability following diet treatment in A (gill), B (liver), and C (muscle). Garlic diet; PD: Propolis diet; WD: Wakame diet. Error bars indicate mean ± SD (n = 3). Different superscripts indicate significant differences ($p < 0.05$). IAA: GSH/MDA

3.7. Discussion

Deleterious effects following Cd exposure in fish may become protracted during recuperation, defying the protective capacity offered by endogenous antioxidants. Thus, the ameliorating potentials of dietary supplemented garlic, propolis, and wakame on recuperating fish model Japanese medaka were assessed. Findings from this study support the rich antioxidative merits of the three dietary sources as previously reported in literature (Mosbaha *et al*., 2017; Nishbori *et al*., 2012). These findings were verified by assessing improvement in growth and general wellbeing of fish fed experimental diets, through mean body weight gains and condition factor.

Morphometric-based condition indices are widely used to assess proximate body composition and, collaterally, feeding and living conditions of fish (Hopkins, 1992). Aqua-culturists typically report growth using absolute (g/d), relative (% increase in body weight), and specific growth rates (%/day) (Li *et al*., 2010). The condition factor (K) of a fish reflects physical and biological circumstances and fluctuations by interaction among feeding conditions, parasitic infections and physiological factors (Le Cren, 1951). Thus, body condition provides an alternative to the expensive *in vitro* proximate analyses of tissues (Sutton *et al*., 2000). Results for diet performance trial in this study showed all experimental diets competed favorably with the control diet, supported fish performance and improved fish condition indices. The appreciable growth rate across all treatment groups indicated adequate dietary support for the species. Final K values did not also differ amongst treatment groups, indicating growth conditions were optimum.

In this study, fish were exposed to a nominal concentration of 0.3 mg L^{-1} Cd. This concentration which was environmentally relevant was verified through preliminary experiments as sub-lethal for fish in this study. As expected, it was sufficient to concentrate within tissues and elicit toxic effects in study fish, so that ameliorating effects of experimental diets can be assessed.

According to Cakmak *et al*. (1993) there is good evidence that the alleviation of oxidative damage and increased resistance to environmental stresses is often correlated with an efficient antioxidative system. Thus, antioxidants performance was included in the battery of bioassays to monitor fish health in the current study. To counteract the adverse effects of ROS, fish have a well-developed antioxidant defense system, which includes two antioxidant categories: enzymatic (e.g., SOD, GR and GPx) and non-enzymatic (e.g., GSH) (El-Gazzar *et al*., 2014) and MT.

SOD is an important, indispensable first line defense antioxidant (Ighodaro and Akinloye, 2018), converting the superoxide anion radical (O_2^-) in ROS to hydrogen peroxide (H_2O_2) and oxygen (O_2). Results from this study showed an initial elevation and subsequent reduction in SOD activity in all studied tissues, following Cd exposure. This observed trend may be due to the overproduction of ROS upon Cd exposure which led to the decline. Similar trend in antioxidant depletion was also reported in the common carp (*Cyprinus carpio*) following Cd exposure (Mehrpak *et al*., 2015; Banaee *et al*., 2015). Declined SOD activities were significantly restored across all studied tissues and diet groups, thus supporting the rich antioxidative potentials of the test dietary sources.

On the other hand, GSH has the function of "master antioxidant" in all tissues. Its central role in the control of many processes such as protein folding, antiviral defense, immune response and detoxification is highlighted by the high concentration of its reduced form (milli molar) (Silvagno *et al*., 2020; Forman *et al*., 2009). Indeed, with upregulated oxidative stress, malnutrition or increased toxic burden due to exposure to environmental contaminants, there can be even greater need for glutathione (Feloi *et al*., 2006; Lopez-Lopez *et al*., 2016). Results from this study clearly showed dietary treatments induced significantly higher GSH contents, relative to those during Cd exposure. Expression levels for GD, PD and WD were superior to those of MD, with GD ranking highest. may have contributed Thus, the antioxidative support of the test dietary sources upon Cd exposure is verified by findings from this study, with GD being more active in reducing Cd induced toxicity.

Correlation analysis between SOD and GSH on the other hand revealed the possible relative antioxidative status induced by the diets in studied tissues. It is known that SOD offers a first line antioxidative defense against ROS and free radical induced oxidative damage. A strong positive correlation between SOD and GSH in the liver tissues, in contrast to gill and muscle tissues in this study corroborates greater antioxidant activity in the liver.

Lipids are responsible for maintaining the integrity of cellular membranes. Extensive peroxidation of lipids (by ROS and free radicals) alters the assembly, composition, structure, and dynamics of lipid membranes (Gaschler and Stockwell, 2017). On the other

hand, antioxidants have a protective effect against tissue injuries in the pathogenesis of which LPO may be involved (Bhadauria *et al*., 2008). Thus, LPO and activity of antioxidant enzymes are the potential biomarkers of pollutant exposure in different organisms (Bechard *et al*., 2008). Generally, lipid peroxide levels *in vivo* may increase, reflecting the physiological conditions induced by oxidative stress such as pathological conditions and/or tissue injuries (Yagi, 1993; Halliwell and Gutterridge, 1989; Cross *et al*., 1987). Results from this study revealed a significant increase in LPO across all assessed tissues following Cd exposure. Magnitudes of LPO were highest and least in gill and muscle tissues, respectively. Dietary treatment however significantly reduced tissue LPO levels in the order GD < WD = PD < MD. This may be attributable to the antioxidative ameliorating effects of the experimental diets. Polyphenols and vitamins are important antioxidants with ability to scavenge ROS (Sandoval-Acuña *et al*., 2014). The high reduction of Cd induced toxicity by GD may be attributable to its rich polyphenol and vitamin profile. On the other hand, the rich amino acid profile of WD played significant role in Cd toxicity reduction and is worthy of note. Some studies have also reported dietary induced amelioration of LPO by heavy metals. For example, Talas *et al*. (2014) reported significantly reduced MDA levels in arsenic (As) exposed carp fish (*Cyprinus carpio*) treated with propolis. Aged garlic extracts (AGE) also caused a significant reduction in LPO in metabolic syndrome rats (Perez-Torres *et al*., 2016).

3.8. Conclusion

Results from this study demonstrated the supportive roles of the experimental diets in enhancing recuperation of subjects from Cd induced toxicity effects. Reduced protective actions of endogenous antioxidants against Cd induced oxidative stress were significantly upregulated following dietary treatments. The antioxidative support of SOD and GSH were restored in subjects fed experimental diets. Cd induced toxicity observed through increased LPO was also significantly reduced in subjects fed experimental diets. These findings corroborate the rich immune stimulating and antioxidative potentials of garlic, propolis, and wakame previously reported by other studies. Overall, dietary supplementation significantly increased recuperation and tissue functions in juvenile medaka fish, with an observed trend of GD > WD = PD > MD. Therefore, GD, PD, and WD may be considered as viable candidates in enhancing amelioration from Cd toxicity effects and enhance recuperation in Cd exposed fish.

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Chapter 4: General discussion, summary and conclusion, and recommendations

4.1. General discussion, summary and conclusion

According to the 2018 edition of the United Nations World Water Development Report, water scarcity, with respect to increasing demand for water, reduction of water resources, and increasing pollution of water, driven by dramatic population and economic growth has been projected to affect nearly 6 billion people globally by 2050 (Boretti and Rosa, 2019). On the other hand, the growing world population produces increasing amounts of toxins which accumulate in water, air, and soil (Häder, 2013). More and more materials are tapped from the environment to sustain the drive and fund raw materials for industrialization. Rather than decrease, water pollution will then intensify over the next few decades and become a serious threat to sustainable development (Veolia/IFPRI, 2016). Thus, the complete abatement of water pollution problems may not yet be in sight, nevertheless, studies are ongoing to bring sustainable solutions. To this end, nature based solutions have been projected (WWDR, 2018). These nature based solutions can protect, manage and restore natural or modified ecosystems (Oral *et al*., 2020).

In this research, the remediating potentials of nature based plants and plant based materials on Cd uptake and toxicity in the medaka fish was explored. Garlic, propolis, and wakame are nature based food materials almost indigenous in all parts of the globe. They are widely grown and produced all year round, and easily affordable. Importantly, their reported pharmacological/antioxidative properties in various studies – polyphenols, vitamins, and amino acids formed a basis for their use in the present study. The food materials as dietary supplements were demonstrated to reduce Cd toxicity and improve fish recuperation from Cd exposure, with reference to objectives of the study.

Chapter 1 provided a background for the study. Here, assessment of environmental and safety risks of exposure to Cd in fish was done. It was shown that Cd exposure in different aquatic environments is ongoing, following mining and industrial activities. Far reaching impacts on aquaculture facilities were also projected. Leaching and washing down of Cd and other heavy metals into aquatic bodies can occur at various times. Interestingly, they are neither degraded nor broken down, and are known to be persistent and cumulative in biological tissues, with long half-life. Their exposure may also be acute or chronic. Variations in exposure conditions may cause delayed toxicity signs in exposed fish, during which period, uptake, concentration, and biomagnification processes in aquatic food chain may continue unnoticed. Consequently, affected fish which are harvested for consumption constitute varying degrees of health risk to the fish consumer.

Study gaps identified included more emphasis on water remediation methods, as shown in the plethora of published research, with little or no information on fish welfare after such

remediation. Few data available on heavy metal contamination and fish treatment were biased towards co-exposure of heavy metal and treatment substance. Others yet, were focused on *in vitro* studies, with little attention to possible confounding factors *in vivo*. To fill these gaps, the present study considered natural scenarios where exposure to Cd may not indicate immediate and obvious signs of toxicity *in vivo*. Furthermore, fish welfare and recuperation strategies following pre-exposure and water treatment were considered in the current study. These considerations gave rise to four objectives of the study, reported in chapter one.

Chapter 2 assessed the first two objectives of the study, which bothered on Cd uptake and depuration, and magnitude of enhanced Cd depuration on account of the experimental diets. Experimental diets were shown to reduce tissue Cd concentration, in the general order of GD > WD = PD > MD, while supporting the upregulation of the Cd binding proteins MT. The findings of this chapter bear special relevance to reduction of tissue Cd burden in exposed fish, and consequently reduction in potential biomagnification in successive links in aquatic food chain or trophic levels.

Chapter 3 assessed the last two objectives of the study, which focused on the amelioration of Cd induced toxicity, as well as the relative magnitude of amelioration in individual experimental diets. Here, the rich antioxidative potentials of the experimental diets were assessed for their ability to up-regulate overwhelmed or depleted endogenous antioxidants and support reduction in Cd induced oxidative stress and toxic effects. Increased oxidative stress may be reflected in increased production of ROS which caused reduced SOD and

GSH observed upon Cd exposure. Increased LPO on the other hand, indicates increased oxidative attack on cells and biomolecules by ROS, which is a manifestation of toxicity effects. Experimental diets therefore improved recuperation by reducing oxidative stress and toxicity effects. Additionally, mortality, which indicates the 'apex' effect of toxicity was also reduced with experimental diets as fish condition improved, observed in the condition indices.

The findings in chapters 2 and 3 may be summarized as shown in figure 22.

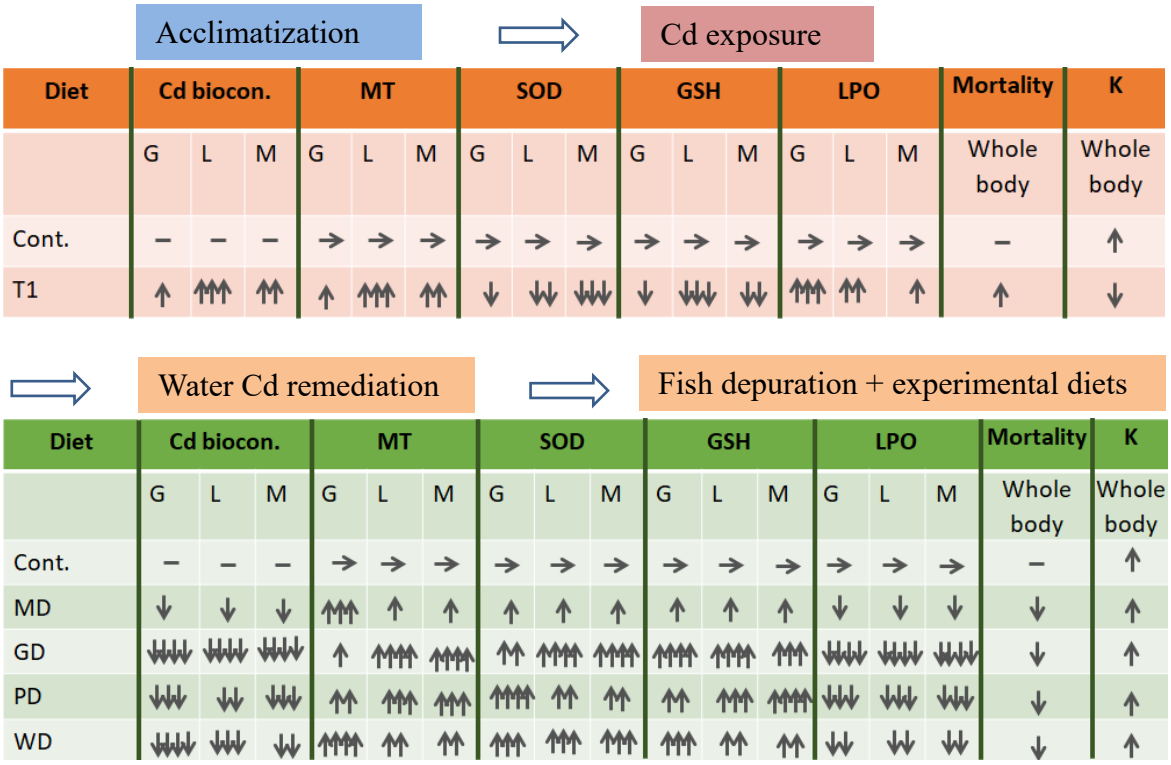

Figure 22. Schematic representation of summary for dietary influence on Cd exposure, bioconcentration, depuration and toxicity reduction in Cd exposed Japanese medaka fish. G: gills, L: liver, M: muscle; MD: medaka diet, GD: garlic diet, PD: propolis diet, WD: wakame diet; Arrow number and orientation represent magnitude and direction of observed effect.

4.2. Recommendations

The findings of this study are relevant in improving fish health status following incidences of Cd pollution and water treatment, especially in aquaculture settings. The diets are relatively cheap, affordable, and readily assessable to all fish famers, all year round, and are strongly recommended to fish farmers. In future studies, concentration and toxicity amelioration with major bioactive compounds *in vitro* are suggested in order to identify the underlying molecular mechanisms of amelioration.

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