Evaluation of Bromophenols in Hong Kong Seafood and Enhancement of Bromophenol Content in an Aquacultured Fish (*Sparus sarba*)

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Abstract

Seafood is one of the most important food resources in Hong Kong. To enhance fishery production, the local government has put much effort into assisting and protecting the local aquaculture industry through technical support and regulation. Aquaculture industry supplied 1770 tonnes of live marine fish for Hong Kong in the year 2000. The distinct flavor of various seafoods attracts people to consume these products regardless of whether they are farmed or naturally harvested. However, some consumers insist that there is an obvious difference in flavor between aquacultured and wild-harvested seafoods. Recent reports have demonstrated that bromophenols, including 2-bromophenol, 4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol and 2,4,6-tribromophenol, could provide desirable and characteristic marine- or brine-like flavor in the wild-harvested seafood. The lack of these flavors in the aquacultured animals is believed to be due to the low concentrations of bromophenols. Should the flavor quality be improved, the market value of the aquacultured animals may increase. The aims of this study are to provide information on the bromophenol content of selected seafood in Hong Kong and to develop a method to enhance the bromophenol content in aquacultured animals.

Two marine fishes, two molluscs, two crustaceans and three marine algae

commonly found in Hong Kong were selected and their bromophenol contents were evaluated. Samples were extracted by simultaneous steam distillation – solvent extraction and analyzed by a gas chromatography/mass spectrometry. The bromophenol contents in the aquacultured seabream (*Sparus sarba*) were enhanced by feeding them with a feed formulation containing seaweeds that possessed high bromophenol contents. The enhancement effects were evaluated by monitoring the changes of bromophenol contents and by sensory evaluation.

Results showed that bromophenols were present in all the selected marine seafoods and seaweeds from Hong Kong. Bromophenol contents of seafoods and seaweeds showed similar seasonal variations, with high content being detected in winter and low in summer. 2,4,6-Tribromophenol was the most abundant bromophenol found among the samples. The concentrations of bromophenols detected in marine animals, e.g. fish, were higher in the gut than in the flesh, and were the highest concentration in seaweeds. These observations implied that diet was a major route to for accumulation of bromophenols, and seaweeds might be the main supply of bromophenol found in marine animals. Therefore, seaweeds were used to develop the bromophenol-rich fish feeds. Fish feed containing 30% *Sargassum siliquastrum* was prepared and found to significantly increase the bromophenol contents in the flesh of fish and affect the flavor

of the aquacultured fish flesh as judged by sensory panelists.

This study quantified the concentration of bromophenols present in the marine organisms found in Hong Kong and provided a potential way to improve the flavor quality of the aquacultured products.

摘要

海產是香港的重要食糧之一。為提高海產的供應，香港政府提供了不同程度的援助及監管措施，以增加本港水產養殖業的產量。在 2000 年，水產養殖業為本港提供了 1770 噸海水活魚。各類海鮮的獨特風味乃其吸引之處，但有些食家卻認為養殖的海鮮與自然捕獲的海鮮在風味上是有所差異。近年來有關海鮮風味的研究，發現海產內的溴代苯酚是一組重要的風味化合物。這些化合物在海產內提供了〝海洋〞及〝海鮮〞的風味。由於這些化合物在養殖的海鮮中的含量較低，令這些人工養殖的海產缺乏了這種重要風味。為提高水產養殖海鮮的質素，其溴代苯酚的含量必須提高。

本研究旨在於調查在本港常見的海產中的溴代苯酚含量，以及找出能夠提高溴代苯酚含量的方法。

研究中選用海產包括兩種海魚、兩種軟體動物、兩種甲殼類動物和三種海藻作樣本，用同步蒸餾及溶劑抽提法抽取溴代苯酚并用氣相色譜質譜測定法分析其含量和質量。此外，還研製了一些含有海藻的新配方魚糧，以用作餵飼鯛魚(*Sparus sarba*)。此種魚糧之效用，會通過調查鯛魚中的溴代苯酚含量以及食品味覺測試來評估。

研究結果顯示，溴代苯酚出現於所有在香港捕獲的海鮮及海藻樣本中，其中以 2,4,6-三溴代苯酚的含量為最高。一般而言，海藻含有

較高的溴代苯酚含量。在海鮮中，消化道的溴代苯酚含量較其肉為高。海鮮和海藻的溴代苯酚含量有著近似的季節性變化，這些現象說明了海鮮的溴代苯酚極可能是由其食物中吸取和累積而來，相信海藻是這些化合物的主要來源。因此，可以把海藻加入魚糧中，以提高水產養殖海鮮的溴代苯酚含量。魚糧投餵結果顯示，加入了百分之三十的褐藻馬尾藻(*Sargassum siliquastrum*)的魚糧能有效地增加水產養殖鯛魚的溴代苯酚含量，并且改善其風味。

此研究對於本港海產的溴代苯酚含量提供了參考資料，亦找出了有效的方法，以提高養殖水產的溴代苯酚含量，令其風味得以改善。

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Contents

Abbreviations

2-BP	2-bromophenol
4-BP	4-bromophenol
2,4-DBP	2,4-dibromophenol
2,6-DBP	2,6-dibromphenol
2,4,6-TBP	2,4,6-tribromophenol
TBC	Total bromophenol content

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Chapter 1

Introduction

Seafood is defined as "those forms of life that reside wholly or in part in an aquatic environment and are regularly consumed as tablefare" (Josephson, 1991). Seafood, including fishes, crustaceans, mollusks and algae, is an important food source for many countries to provide proteins and minerals (Nybakken,1997). Besides, the distinct flavor attracts people to consume seafood. Fresh seafood can be prepared in various ways such as steaming and boiling to make different dishes (Chung *et al.*, 2001). Furthermore, seafood can be processed by salting, drying, smoking, pickling, and even fermentation to produce seafood products (Horner, 1997). Salted-fish, oyster sauce and shrimp paste are some of the fermentation products commonly used in Chinese cuisine (Montano *et al.*, 2001).

Traditionally, supply of seafood depends mainly on the catch from the wild (Chen, 2001). In the last decade, the wild fish population has declined dramatically due to overexploitation (Nyakken, 1997). Productivity in the major marine fishing areas, including the northwest Pacific Ocean, has declined significantly (Weber, 1994). In order to solve this problem, fishermen have started to cultivate various

marine and estuarine plants and animals. By carrying out such aquaculture (Nyakken, 1997), it is hoped that the amount of food supply from marine sources can be increased.

However, problems do exist in aquaculture. One of the major problems is that the disease resistance of the fish population is usually low because of captive or crowded conditions (Mazzola and Rallo, 1981; Nyakken, 1997). It is also difficult to maintain the quality of water in ponds or tanks in proper physical and chemical conditions because wastes and toxic materials are continuously being excreted by the cultured organisms (Nyakken, 1997). Many marine animals cannot complete their life-cycle in captivity. For example, some serranid species, which have high economic values, must be treated with suitable hormones in order to spawn in captive environment (Hassin *et al.*, 1997). A possible reason for infertility in the adult is the abnormal photoperiod. It was shown that photoperiod is crucial in the development and maturation of gonads (Roberts *et al.*, 1978; Hempel, 1979; Hansen *et al.*, 2001). Though few studies on the reproduction of marine animals have succeeded and reached market application, many aquacultured animals still rely upon for natural seed and fingerlings (Moriwake *et al.*, 2001). Apart from the production problems, the flavor quality of aquacultured seafood is also frequently criticized

(Sylvia and Graham, 1991; Kummer, 1992). In recent years, there have been a number of studies on the differences in flavor between wild-harvested and aquacultured seafood, and the ways to improve the flavor of the aquacultured products.

Previous studies showed that bromophenols were commonly found in seafoods from different marine sources and were responsible for sea-like and brine-like flavors (Boyle *et al.*, 1992a; b). It was suggested that the subtle differences between the wild-harvested and the aquacultured seafood were caused by the quantitative differences in their bromophenols (Boyle *et al.*, 1993; Whitfield *et al.*, 1997b). Aquacultured animals lack sea-like flavor due to their relatively low concentrations of bromophenols (Whitfield *et al.*, 1997b). A literature review about bromophenols will be provided in the next chapter. The possibility of applying such compounds to enhance the seafood flavor will be discussed.

In Hong Kong, fresh fish is one of the most important primary products (Information Services Department, 2000). Fish are farmed locally to increase the fishery supply. There have been numerous studies performed on the distribution and the possible sources of bromophenols, but no information on such compounds in

the marine species in Hong Kong is available.

In the current study, the main objectives were (1) to investigate the distribution and the seasonal variations of the bromophenols in selected seafoods and seaweeds found in Hong Kong; (2) to develop methods that could increase the concentrations of bromophenols in aquacultured marine fish, and thus to improve their flavor quality.

Chapter 2

Literature review

2.1 Fisheries in Hong Kong

Seafood, including fishes, crustaceans, mollusks and algae, is an important food source. In Hong Kong, fresh fish is one of the most important primary products. According to the Information Services Department, HKSAR (2000), the productions from capture and culture fisheries in 2000 were about 157010 and 4660 tonnes, respectively, valued at HK$1.7 billion.

The capture fishery was manned by 11900 local fishermen and 5200 Mainland deckhands. In the Hong Kong fishing fleet, there were 5250 vessels. The main fishing method was trawling supplying 133458 tonnes of fish in 2000 (Information Services Department, 2000). Fish is caught mainly in the waters around the South China Sea.

The culture fishery supplies both seawater and freshwater fish to the local market. There were 1418 mariculturists operating in 26 designated fish culure zones under license from the Agriculture, Fisheries and Conservation Department,

HKSAR (AFCD) (Information Services Department, 2000). The Aquaculture Fisheries Division of AFCD is responsible for promoting the aquaculture industry through regulations, and provision of technical and financial support (Wei, 1998-1999). In 2000, production of live marine fish from the aquaculture sector was 4660 tonnes valued at $102 million (Information Services Department, 2000).

2.2 Flavor of seafood

The flavor of seafood, including both the volatile and the nonvolatile taste components, is closely related to the consumer acceptance of the seafoods (Wesson *et al.*, 1979; Lindsay, 1990; Josephson, 1991; Shahidi and Cadwallader, 1997). It is affected by the nature of the initial product and conditions during processing and distribution (Boyle *et al.*, 1993; Boyd, 1997). Besides, there are many physiological, seasonal and environmental factors such as feeding patterns, stage of sexual cycle and migration patterns in wild-captured fisheries that affect the flavor quality of the seafood products (Bilinski *et al.*, 1984; Josephson *et al.*, 1991). The nonvolatile taste-active constituents include nucleotides and free amino acids are accompanied by the volatile compounds that produce the distinct seafood flavor (Lindsay, 1994). The volatile components contributed by several classes of compounds are formed in the living species, at the time of death and during food

processing. The flavor of fresh seafood is usually described as sweet, plant-like, metallic and slightly fishy. Some species-specific volatile compounds are responsible for the characteristic flavor of each individual species (Josephson, 1991). Generally, the volatile compounds found in seafood belong to the following categories: lipid-derived volatile aroma compounds, alcohols, aldehydes, ketones, sulfur- and nitrogen- containing compounds, thermally generated compounds and bromophenols.

2.2.1 Lipid-derived volatile aroma compounds

Lipid-derived volatile aroma compounds formed from the polyunsaturated fatty acids play a predominant role in seafood flavor (Josephson, 1991). The enzyme-mediated pathways and autoxidation degradation are the two mechanisms involved.

In the enzyme-mediated pathways, the abundant polyunsaturated fatty acids present in the seafood are converted primarily to fatty acid hydroperoxides via lipoxygenase-mediated reactions and then subsequently converted to numerous volatile aroma compounds (Josephson and Lindsay, 1986; Hsich *et al.*, 1988). The major flavor compounds derived are 6-, 8- and 9-carbon aldehydes, ketones and alcohols (Josephson and Lindsay, 1986) giving fresh-plant-like aroma notes.

The autoxidative degradation of polyunsaturated fatty acids produces volatile carbonyls, acids and alcohols. It generates fish-like flavor in fresh fish, whereas it gives oxidized-fish flavor to frozen seafood (Josephson, 1991).

2.2.2 Alcohols, aldehydes and ketones

Unsaturated alcohols, aldehyde and ketones with carbon number less than 10 are important flavor compounds in seafood (Pan and Kuo, 1994). Most of them are products derived from fatty acids. Alcohols have a minor effect on flavor unless they are present in relatively high concentrations. For example, 3-methyl-1-butanol possessed a wine-like odor in fermented shellfish products (Nykänen and Suomalainen, 1983), 3,6-Nonadien-1-ol gives distinct melon-like flavor to oyster (Josephson *et al.*, 1985), and 5*Z*-octa-1,5-dien-3-ol imparts a metallic off-flavor to prawn and sand-lobster (Whitfield *et al.*, 1982).

Aldehydes have low odor detection threshold values, which make them important flavor compounds in seafood. Examples include (*E*,*Z*)-2,6-nonadienal found in pickled fish and fresh oyster which possesses cucumber-like flavor (Josephson *et al.*, 1985; 1987), and benaldehyde found in crayfish tail meat which

gives a creamy and nutty flavor (Vejaphan *et al.*, 1988; Tanchotikul and Hsieh, 1989).

Ketones are another group of flavor compounds found in seafood. 2,3-Pentanedione gives an intense buttery note to oyster (Josephson *et al.*, 1985). 2,3-Butadione is important in crab meats, giving sour or creamy aroma (Chung and Cadwallader, 1994; Chung, 1999).

2.2.3 Enzymatic conversion of sulfur- and nitrogen-containing precursors

Several sulfur-containing compounds such as dimethyl sulfide and dimethyl disulfide produced by endogenous enzyme systems were reported to be associated with the laver-like or petroleum flavor (Tokunaga *et al.*, 1977; Shiomi *et al*, 1982). Trimethylamine formed from bacterial reduction of trimethylamine oxide is associated with fishy smell (Ackman, 1976; Laycock and Regler, 1971). This compound is abundant in deteriorated marine fish and seafood.

2.2.4 Thermally generated compounds

Flavor compounds such as pyrazines, pyridines, thiophene and thiazole are generally produced by pyrolysis reactions and Maillard reactions via Strecker

degradation during heating (Whitfield, 1992; Fors, 1983). Pyrazines are important flavor contributors giving nutty and roasted notes to a variety of seafood such as blue crab, crayfish and dried scallop (Chung and Cadwallader, 1993; Baek and Cadwallader, 1996; Chung *et al.*, 2001).

2.2.5 Bromophenols

Whitfield *et al.* (1988) first discovered that elevated amounts of bromophenols, particularly 2,6-dibromophenol, were the cause of iodoform-like or iodine-like off-flavor in Australian prawns. Recently, scientists discovered that bromophenols are a group of key flavor compounds present in most seafood. They are widely distributed in marine fish, crustaceans, mollusks, etc (Boyle *et al.*, 1992a). These compounds can provide desirable sea- or brine-like flavor, and are suggested to be able to enhance the intensity of the existing flavor of seafood (Boyle *et al.*, 1992a; 1992b; Whitfield *et al.*, 1998). The monobromophenols enhance sweetness and overall flavor of seafood; 2,6-dibromophenol and 2,4,6-tribromophenol produce desirable prawn-like and ocean-like flavor (Boyle *et al.*, 1992b; Whitfield *et al.*, 1996). However, in some of the Australian prawns studied by Whitfield *et al.* (1988), the concentration of 2,6-dibromophenol exceeded 200 μg/kg fresh weight. This is in great excess of its threshold value (0.0005 *n*g/g in water). It led to the

sensation of iodoform-like off-flavor.

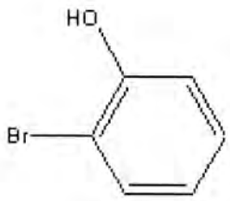
2.2.5.1 General properties of bromophenols

There are five major types of the bromophenols including 2-bromophenol (2-BP), 4-bromophenol (4-BP), 2,4-dibromophenol (2,4-DBP), 2,6-dibromophenol (2,6-DBP) and 2,4,6-tribromophenol (2,4,6-TBP). Their structures are shown in Figure 2.1 (p.12).

The general properties of bromophenols are listed in Table 2.1 (p.13). Due to the presence of heavy bromine atoms, the molecular weights of the bromophenols are relatively high when compared with other flavor compounds. High boiling point of each of these compounds makes their isolation from seafood more difficult (Boyle, 1993). The problem is solved by the use of simultaneous steam distillation-solvent extraction method (SDE) which provides adequate recoveries for quantitative analysis by gas chromatography/mass spectrometry (GC/MS) (Whitfield *et al.*, 1988; Boyle *et al.*, 1992a).

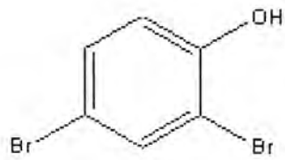
Bromophenols are relatively polar and can disperse to some degree in water (Winholz *et al.*, 1983). However, Boyle *et al.* (1992b) reported that

Figure 2.1. Chemical structures of bromophenols.

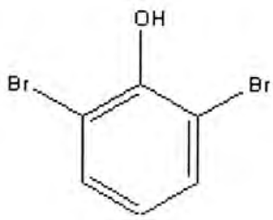


2-bromophenol

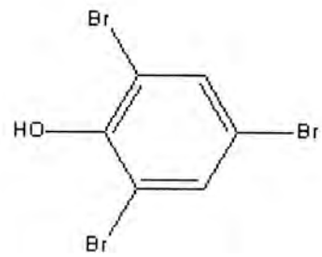
4-bromophenol



2,4-dibromophenol



2,6-dibromophenol



2,4,6-tribromophenol

Table 2.1. General chemical and physical properties of volatile bromophenols.

(From Boyle *et al.*, 1993)

	Bromophenols[a]					
Property	2-BP	4-BP	2,4-DBP	2,6-DBP	2,4,6-TBP	Reference[b]
Molecular weight (g/mole)	173	173	252	252	330	1,2
Boiling point (°C)	194	238	154	162	244	1,2
Solubility	Water, ether	Alcohol, $CHCl_3$, ether, 7 parts water	Ether, alcohol	Ether, alcohol	Alcohol, $CHCl_3$, 14,000 parts water	3
Log P	1.69	1.94	3.00	2.37	3.74	4

[a] BP = bromophenol; DBP = dibromophenol; TBP = tribromophenol; Log P = experimentally derived octanol-water partition coefficients.

[b] 1: Buckingham, 1982; 2: Verschueren, 1983; 3: Winholz *et al.*, 1983; 4: Boyle *et al.*, 1992a.

monobromophenols did not disperse in vegetable oil. Considering the octanol/water partition coefficients, Log P, [Table 2.1 (p.13)] reported by Boyle *et al.* (1992a), the values ranged from below 2.0 for monobromophenols to 3.74 for tribromophenol. When the Log P values of bromophenols were less than 6, they were readily absorbed by fish (Gobas, 1988). Usually bioconcentration occurs in those compounds with Log P $\geq$ 3.0, (Poels *et al.*, 1988). While none of them seems to be able to strongly bioaccumulate (Heil and Lindsay, 1989), only 2,4-dibromophenol and 2,4,6-tribromophenol have Log P = 3.0 and 3.74 respectively, and are more likely to be bioaccumulated in seafood. The bromophenols may be solubilized and lost into the aqueous phase. The presence of bromophenols in the seafood tissue reflects the equilibrium between dietary intake and normal depuration (Boyle *et al.*, 1993). Elevated bromophenol levels due to excess dietary intakes would result in iodoform-like off-flavor (Bemelmans and den Braber, 1983; Whitfield *et al.*, 1988). However, ingestion of nominal concentrations of bromophenols contributes to the natural sea-, iodine-, and marine-like flavors (Boyle *et al.*, 1992b).

2.2.5.2 Thresholds of bromophenols

The contribution of a flavor compound depends on its concentration and its

odor detection threshold value. The flavor characteristics provided by the bromophenols in seafood vary dramatically with the presence of isomers, their concentrations and the medium they are in (Bemelmans and den Braber, 1983; Whitfield *et al.*, 1988; Boyle *et al.*, 1992b). The threshold values of the bromophenols in water and in prawns were determined by some scientists [Table 2.2 (p.16)].

Bromophenols are powerful flavor compounds with relatively low threshold values in water. Whitfield *et al.* (1988) reported that 2,6-dibromophenol with the concentration up to 250 *n*g/g in the off-flavored prawns were in great excess of the threshold (0.0005 *n*g/g in water) causing the iodoform-like off-flavors. This unpleasant flavor was absent at concentration below 32 *n*g/g. Most of the bromophenols provide chemical, iodine- or phenolic-like flavor when evaluated in water.

When bromophenols are mixed with fish, shrimp or triglyceride oils, they give characteristic flavor with different isomers (Boyle *et al.*, 1992b). 2,6-Dibromophenol was described as iodine- and shrimp-like and 2,4,6-tribromophenol gave saltwater fish- and brine-like notes. 2-Bromophenol was reported to be able to

Table 2.2. Flavor threshold concentrations of bromophenols. (From Whitfield *et al.*, 1988).

Compound	Water		Prawn meat	
	Threshold (μg/kg)	Flavor description	Threshold (μg/kg)	Flavor description
2-bromophenol	3×10^{-2}	Phenolic/iodine	2	Phenolic
4-bromopehnol	23	Phenolic	nd	nd
2,4-dibromopehnol	4	Phenolic	nd	nd
2,6-dibromopehnol	5×10^{-4}	Iodoform	6×10^{-2}	Iodoform
2,4,6-tribromopehnol	6×10^{-1}	Iodoform	nd	nd

nd = Not determined

enhance rich marine seafood flavor characteristics (Boyle *et al.*, 1992b). While 2,6-dibromophenol and 2,4,6-triboromophenol were perceived as fishy-like when evaluated in vegetable oil. Marine herring-like oil flavor was detected when these bromophenols were evaluated in deodorized menhaden oil (Boyle *et al.*, 1992b).

Desirable sea-like, sea salt-like and sea fish-like flavor were produced when 2-BP, 2,6-DBP,or 2,4,6-TBP were incorporated into bland marinated whitefish. The concentration of 2-BP at 10 *n*g/g produces rich, full and sea-like flavor; that of 2,6-DBP at 0.1 *n*g/g gives crab- or shrimp-like flavor; that of 2,4,6-TBP at 10 *n*g/g produces sea salt- or sea fish-like flavor (Boyle *et al.*, 1992b). Seafood containing bromophenols higher than these values would likely possess the desirable sea-like and ocean-like flavor.

2.2.5.3 Toxicity of bromophenols

Bromophenols have been recognized as irritants to skin, eyes and mucous membranes. They are also identified as moderately toxic compounds with an extreme unpleasant odor (Ashworth and Cormier, 1967; Higa *et al.*, 1980; Sax and Lewis, 1989).

4-Bromophenol was found to be a potential experimental tumorigen (Sax and Lewis, 1989). However, no sub-lethal toxic effects have been reported in mice subjected to interperitoneal and oral administration of 2-bromophenol, 3-bromophenol, 2,4-bromophenol and 2,4,6-tribromophenol (Sweet, 1987). The LD_{50} of 2,4,6-tribromophenol in rat was 200 mg/kg administrated orally and this dose is approximately 200 to 500 times greater than the highest concentrations found in Australian crustaceans by Whitfield *et al.* (1988). It is impossible to translate the toxicity results of test species directly to human case. However, estimation can be made on the LD_{50} of bromophenols in human by gathering various data including reported LD_{50} values of bromophenols; the surface area and weight ratios between man and animal bodies. To obtain a respective LD_{50} dosage comparable to rodent, an adult human will have to consume 8 million to 600 million prawns. The dosage also depends on the types of bromophenols. Therefore, it is unlikely that bromophenols, either naturally obtained or artificially incorporated, would create an acute toxicological effect (Boyle *et al.*, 1993). However, additional tests should be performed to investigate the sub-lethal toxicity and the adverse effects of these compounds on the marine fauna, flora, and environment when they are applied in actual aquaculture.

2.2.5.4 Previous studies about bromophenols

The studies about the importance of bromophenols as flavor compounds in seafood began when Whitfield *et al.* (1988) discovered an elevated concentration of them causing an iodoform-like off-flavor in Australian prawns. In 1992, Boyle *et al.* (1992a; b) identified bromophenols as the key flavor components providing desirable sea- and brine-like flavor in seafood. They were widely distributed in marine fishes and seafoods. They also found that bromophenols were accumulated from diet via the food chain. In fact, freshwater fish lack bromophenols as freshwater environment lacks bromine, and the corresponding freshwater flora and fauna are incapable of biosynthesizing bromophenols (Fenical, 1981). It seems that the presence of bromophenols is a key factor to differentiate the flavor between the species from marine and freshwater sources. Further studies were carried out on the distribution of the bromophenols in different marine species from different locations (Whitfield *et al.*, 1997b; 1998), but no information about such compounds in the marine species in Hong Kong are available.

In the current study, the distribution of bromophenols in selected seafoods from seawater and freshwater origin in Hong Kong were investigated. The effects of these compounds on the flavor of the seafoods studied were discussed.

2.2.5.5 Bromophenols in aquacultured seafood

The study on the Australian wild-harvested and cultivated prawns showed that the total bromophenol contents of the wild-harvested prawns (9.5-1114 *n*g/g) were much higher than those of the cultivated ones (<1 *n*g/g) (Whitfield *et al.*, 1997b). Sensory evaluations showed that the meat of the wild-harvested prawns possessed briny, ocean-like and prawn-like flavors whereas the cultivated ones were described as bland. It was believed that the subtle differences between the wild-harvested and the cultivated seafood were caused by the quantitative difference in their bromophenols (Whitfield *et al.*, 1997b). The possibility of increasing the bromophenol contents by adjusting the dietary sources was suggested (Whitfield *et al.*, 1997a).

In Hong Kong, aquacultured seafood is supplied to the market to increase fishery production. In our study, the bromophenol contents in the aquacultured fish were investigated and compared with that in the wild-harvested fish.

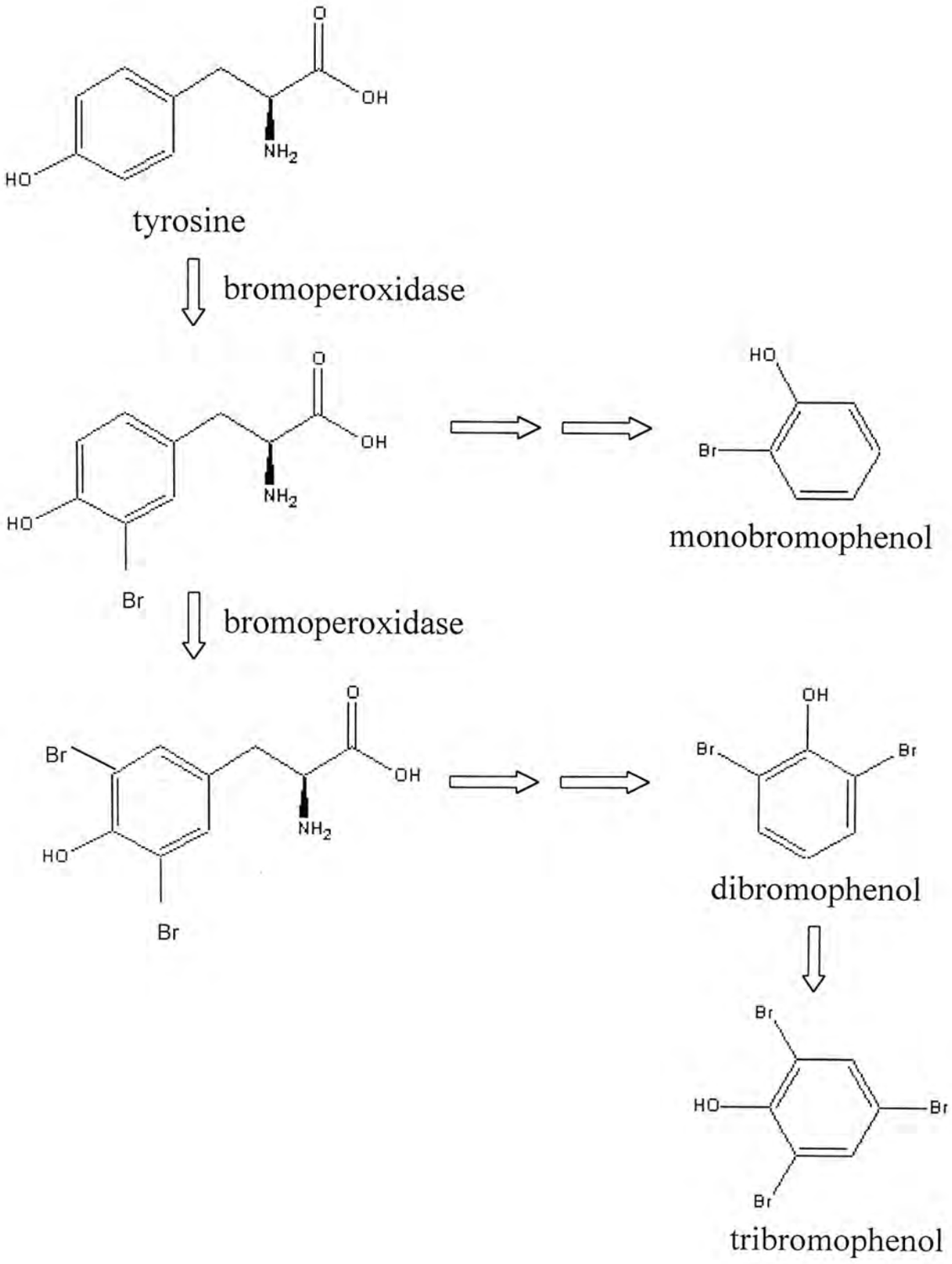
2.2.5.6 Possible dietary sources of bromophenols

The investigation on marine algae and polychaetes were performed to find out

the possible dietary sources and the production mechanisms of such compounds (Flodin and Whitfield, 1999a; 1999b; Whitfield *et al.*, 1999a; 1999b). The results showed that the bromophenols were generally detected at relatively high concentrations in Australian marine algae (0.9 – 2590 *n*g/g) and polychaetes (58 – 8.3 million *n*g/g). They were considered to be dietary sources of the bromophenols in seafood (Whitfield *et al.*, 1999a; 1999b).

High levels of bromophenols were detected in marine algae because they contain bromoperoxidases which are the enzymes involved in the biosynthesis of bromophenols. It was believed that bromophenol and lanosol shared the same precursor, tyrosine, by marine algae (Flodin and Whitfield, 1999a). The biosynthetic pathway from tyrosine to bromophenol was thought to be similar to that of the synthesis of lanosol (Manley and Chapman, 1978). Lanosol (2,3-dibromo-4,5-dihydroxybenzyl alcohol) is commonly found in marine algae (DeBusk *et al.*, 2000) as feeding deterrent. It was also the potential cause of disinfectant taint in foods and pollutant (Höfer, 1998; Adams *et al.*, 1999). In the formation of bromophenols, it is hypothesized that tyrosine is brominated by bromoperoxidase [Figure 2.2 (p.22)] and the reaction involves electrophilic substitution of bromine onto tyrosine in the presence of hydrogen peroxide (Higa *et al.*, 1980). During the

Figure 2.2 Possible biosynthetic pathway of bromophenol in marine algae (From Boyle *et al.*, 1993).



reaction, tyrosine was deaminated, successively oxidized and decarboxylated to yield 4-hydrobenzaldehyde which can be either oxidized to 4-hydrozybenzoic acid or reduced to 4-hydroxybenzyl alcohol. Then, bromoperoxidases in algae catalyze the bromination of these compound to mixtures of 2-BP, 4-BP, 2,4-DBP, 2,6-DBP and 2,4,6-DBP (Flodin and Whitfield, 1999a). Several intermediates of the above-mentioned compounds and bromoperoxidase have been identified in *Ulva lactuca*, an observation that strongly supported this hypothesis (Flodin and Whitfield, 1999b).

In the current study, the distributions of bromophenols in marine algae commonly found in Hong Kong were studied to evaluate if they are possible sources of bromophenols in marine environment.

2.2.5.7 Possibility of increasing bromophenol contents in aquacultured fish

To improve the quality of the aquacultured seafoods, their flavor should be improved. Previous studies showed that the differences between the flavors of wild-harvested and the cultivated seafood were caused, in part, by the quantitative difference in bromophenols (Whitfield *et al.*, 1997b). It might be possible to improve the flavor quality of the aquacultured seafood by increasing the bromophenol contents to suitable levels. Marine algae containing relatively high

concentrations of bromophenol could be used as bromophenol-rich dietary sources (Whitfield *et al.*, 1999b). In the current study, methods would be developed and tested to increase the bromophenol concentrations in aquacultured fish by incorporating marine algae into the fish feeds.

Marine algae were widely utilized to produce medicine, food additives, and chemicals for industrial uses (Hodgkiss and Lee, 1983). In this project, they were selected to produce fish feeds because (1) they contain relatively high concentrations of bromophenols (Whitfield *et al.*, 1999b); (2) they grow abundantly in the waters of Hong Kong (Hodgkiss and Lee, 1983) so samples can be readily collected; and (3) they are potential dietary sources of protein and lipid for fish (Wahbeh, 1997). The fish feeds developed in this experiment would be used to feed the experimental fish. The effects of these feeds would be evaluated by monitoring the bromophenol content of, and by sensory evaluations on the meat of fish fed with them.

2.3 Criteria for selecting experimental fish model

Members of Sparidae are important economic fish for food in many countries (Lau and Li, 2000). They are commonly known as seabream or porgy, with a vertically flattened body and strong spiny rays in the dorsal fin (Girin, 1983).

European gilthead seabream (*Sparus aurata*) has been extensively farmed throughout the Mediterranean (Pitt *et al.*, 1977). Yellow finned black porgy (*Acanthopagrus latus*) and silvery black porgy (*Acanthopagrus cuvieri*) are reared in Kuwait, while West Altantic seabream (*Archosargus rhomboidalis*) has been cultured in Florida (Houde and Potoff, 1976; Dowd and Houde, 1980). In the Western Pacific areas like Japan, Hong Kong and Taiwan, the seabream industry has also flourished. Red seabream (*Pagrus major*), black seabream (*Mylio macrocephalus*) and silver or gold-lined seabream (*Sparus sarba*) are major species for aquaculture (Girin, 1983). Besides the economic importance, a considerable amount of scientific information is also available on seabream. Research on seabream regarding general culture practices (Sanders, 1975; Cuyvers, 1979), disease (Masumura and Wakabayashi, 1977; Colorni *et al.* 1981), growth and life history (Chang and Chen, 1972; Zhang *et al.*, 1980), larval rearing and reproduction (Billard 1978; Girin and Devauchelle, 1978; Iwata *et al.*, 1978), nutrition (Zohar and Gordin, 1979; Lee *et al*, 1981; Woo and Kelly, 1995) and physiology (Woo and Fung, 1981; Woo and Wu, 1982) are readily found.

Therefore, silver seabream (*Sparus sarba*) was chosen as the experimental model in the present study because (1) it is a typical marine fish with high economic

value (HK$ 30 per 300 g fish); (2) it is readily available from local fish farms; (3) local laboratory facilities are able to rear them in a healthy condition; and (4) previous studies have developed formulated diets that effectively keep fish growing (Woo and Kelly, 1995).

Chapter 3

Distributions of bromophenols in selected Hong Kong seafoods

3.1 Introduction

Seafood is one of the most important food sources in Hong Kong. Many consumers appreciate their delicate flavors. Previous studies focused on the characteristic flavor of seafood contributed by microbial degradation, lipid derived and thermally generated compounds (Josephson, 1991; Josephson and Lindsay, 1986; Josephson *et al.*, 1983; 1984; Kuo and Pan, 1991). Recently, researchers found that bromophenols, including 2-bromophenol (2-BP), 4-bromophenol (4-BP), 2,4-dibromephenol (2,4-DBP), 2,6-dibromophenol (2,6-DBP) and 2,4,6-tribromophenol (2,4,6-TBP) were another group of key flavor compounds found in seafood (Whitefield *et al.*, 1988, 1997a, b; Boyle *et al.*, 1992a, b). Among them, the most potent compounds were 2-BP, 2,6-DBP and 2,4,6-TBP with the flavor threshold at 3 $\times 10^{-2}$, 5 $\times 10^{-4}$ and 0.6 ng/g in water, respectively (Whitfield *et al.*, 1988). They were reported to produce desirable marine- or ocean-like flavor as well as to enhance the intensity of existing flavor in seafood (Boyle *et al.*, 1992a,b; Whitfield *et al.*, 1997b). Previous researches have been carried out on the distributions of these compounds in different kinds of seafoods such as prawns, salmons and fishes (Boyle

et al., 1992a; Whitfield *et al.*, 1997b; 1998). It was found that the bromophenols were widely distributed in seafoods and were virtually absent in freshwater fish (Boyle *et al.*, 1992a).

In Hong Kong, research on the flavor of seafood has been quite scarce even though the consumption of seafood is popular. Studies on the volatile components in Hong Kong seafood are limited to crab and dried scallop (Chung, 1999; Chung *et al.*, 2001; 2002). However, specific information on the presence and levels of bromophenols in seafoods found in Hong Kong are unavailable. Therefore, the objectives of this survey were to quantify the amounts of bromophenols in selected marine fishes and seafoods, and to compare their bromophenol levels with those found in a popular freshwater fish.

3.2 Materials and methods

3.2.1 Sample collection and preparation

Seafoods including marine fishes, mollusks and crustaceans were studied. Live marine fishes including rabbitfish (*Siganus canaliculatus*) and brown-spotted grouper (*Epinephelus areolatus*) were bought from local market. For each species, samples were purchased from the same shop throughout the study. Each fish was

divided into 2 parts including the gut and the flesh (separated from the head, body and backbone). Both gut and flesh of each fish were collected whereas the head, body and backbone were discarded.

Similarly, live molluscs including clam (*Tape philippinarum*) and oyster (*Ostrea rivularis*), and crustaceans including shrimp (*Penaeus japonicus*) and crab (*Charybdis feriatus*) were purchased. Both clams and crabs were steamed for 15 minutes within one hour of purchase. Their meats were picked manually. Each shrimp was carefully divided into head (cephalothorax) and body (abdomen). Seafood samples were collected every two months from December, 1999 through December, 2000. Freshwater fish, grass carp (*Ctenopharyngodon idellus*), was obtained from local market in November, 1999.

Organic solvents, pentane and diethyl ether, were purchased from Lab-scan Ltd. (Ireland) with purity of 99% and 99.5% respectively. Standard samples of 2-BP, 2,4-DBP and 2,6-DBP were bought from Aldrich Chemical Co. (Milwaukee, WI), and those of 4-BP and 2,4,6-TBP were purchased from Acros Organics (Belgium). The purities of the five bromophenols ranged from 97% to 99%.

3.2.2 Simultaneous steam distillation-solvent extraction (SDE)

The extraction method was adapted from Whitfield *et al.* (1988, 1997b). Each sample was homogenized by a National Blender (MX-T2GM, Matsushita Electric Co. Ltd., Taipei, Taiwan) for four minutes. Treated samples, 1 mL of internal standard (1,3,5-trimethylbenzene, concentration at 84.7 μg/mL) and 500 mL of boiled double distilled water were transferred to a 5-L round bottom flask. The sample was then acidified to pH 1 with 96% sulphuric acid. Extraction with 40-mL of pentane/diethyl ether (9:1 v/v) for 2.5 hours in a Likens and Nickerson type SDE apparatus (Cat. No. K-523010-0000, Kontes, Vineland, NJ) was then carried out. The pH of the residue after extraction was measured again to ensure that the acidity was maintained throughout the process. Triplicate extractions of each sample were carried out. Extract collected was further concentrated to 0.25 mL with a stream of ultra high purity (99.999%) nitrogen and dried over 2.85 g anhydrous sodium sulfate. The concentrated extract was temporary stored in a 15-mL conical tube at –80°C until further analyses were carried out.

3.2.3 Gas chromatography/mass spectrometry (GC/MS)

A GC/MS system consisting of a Hewlett-Packard 6890 GC coupled with a HP 5973 mass selective detector (MSD) (Hewlett-Packard Co., Palo Alto, CA) was used

for qualitative and quantitative analyses. Five μL of each extract was injected, in splitless mode with injector temperature at 200°C, into a fused silica open tubular column (Supelcowax-10, 60 m length × 0.25 mm i.d. × 0.25 μm film thickness; Supelco, Inc., Bellefonte, PA). Helium gas (ultra high purity grade, 99.999%) was used as carrier gas at constant linear velocity at 30 cm/s. Oven temperature was programmed from 100 to 200°C at a ramp rate of 10°C/min. The initial and final hold times were 5 and 75 mins., respectively. MS interface, ion source and MS quadrupole temperatures were set at 250°C, 230°C and 106°C, respectively. Ionization voltage was 70 eV and electron multiplier voltage was 1200 V. Selected ion monitoring (SIM) GC/MS procedure was used. Ions were monitored for 2- and 4-bromophenol (2- and 4-BP) at *m/z* 172 and 174; for 2,4- and 2,6-dibromophenol (2,4- and 2,6-DBP) at *m/z* 250 and 252; for 2,4,6-tribromophenol (2,4,6-TBP) at *m/z* 330 and 332; and for internal standard 1,3,5-trimethylbenzene at *m/z* 105 and 120 (Lee *et al.*, 1984; Whitfield *et al.*, 1997b; Chung, 1999).

3.2.4 Compound identification and quantification

The presence of each bromophenol was confirmed by the detection of a single peak in the selected ion chromatogram at corresponding retention time and by the presence of the two characteristic ions listed above with particular isotopes ratios

(Whitfield *et al.*, 1997b).

For quantification, 3-point standard curves for each bromophenol were established. Solutions (5mL) containing 5 mg of each of the five bromophenols was prepared. Serial dilutions at ratios of 1:5 and 1:25 were made. A constant amount of internal standard, 1,3,5-trimethylbenzene (5 mg), was added into each of the above solutions prepared. The area ratios from both selected ions (bromophenol/internal standard) were plotted against the amount ratios to obtain a response factor for each bromophenol. Ions chosen were with *m/z* of 172 (monobromophenols), 252 (dibromophenols), 330 (tribromophenol) and 105 (internal standard). When response factor of each compound was obtained, the amount ratios (bromophenol/internal standard) of each bromophenol in the extracts could be calculated. Concentration of a bromophenol in a sample was calculated by the following equation (Equation 1):

Concentration of a bromophenol (*n*g/g dry weight)

$$= \frac{\text{(amount ratio of bromophenol/internal standard)} \times \text{amount of internal standard } (n\text{g})}{\text{dry weight of sample (g)}}$$

.....Equation 1

The total bromophenol content (TBC) was calculated by the following equation (Equation 2):

Total bromophenol content (TBC) (*n*g/g dry weight)

=Summation of the concentrations of 2-BP, 4-BP, 2,4-DBP, 2,6-DBP and 2,4,6-TBP (*n*g/g) .....Equation 2

3.2.5 Recoveries

The extraction efficiencies of the SDE technique were determined by the recoveries of each standard bromophenol from a model system. The values of the recoveries obtained were used to calculate the original amount of bromophenols presented in the samples. They were determined by extracting known amount of each bromophenol with SDE method, and quantified the amount with the GC/MS system under the same experimental conditions as described previously. Ten μg of each of the five bromophenols to a 100-g freshwater grass carp flesh, which did not contain bromophenols. The recovery of each bromophenol was calculated by the following equation (Equation 3):

Recovery (%)

$$= \frac{\text{Concentration of bromophenol detected}}{\text{Concentration of bromophenol standard added}} \times 100\% \quad \text{.....Equation 3}$$

3.2.6 Moisture determination

The percentage moisture of each of the samples was determined using a Mettler LJ16 moisture analyzer (Mettler-Toledo, Switzerland). Dry weights of the samples were then determined. Concentrations of the bromophenols in the samples were expressed on a dry weight basis.

3.2.7 Statistical analysis

Quantities of the total bromophenol content in the gut and the flesh of fishes, and in the heads and bodies of shrimps were compared by *t*-test with computer software (SPSS 10.0, Chicago, IL) at $p<0.05$ level of significance.

3.3 Results and discussion

3.3.1 Distribution of bromophenols in seafoods

In this study, six seafood samples commonly found in the markets in Hong Kong were analyzed to investigate the distribution of the five bromophenols. Samples were obtained every two months throughout the year 1999-2000. The total number of sample collection was seven. The bromophenols in the samples were extracted by the SDE apparatus, and were analyzed and quantified with a GC/MS

system. The average percentage recoveries were 99.7 ± 0.4% for 2-BP, 38.0 ± 3.2% for 4-BP, 99.3 ± 0.9% for 2,4-DBP, 93.1 ± 0.3% for 2,6-DBP and 62.5 ± 7.8% for 2,4,6-TBP.

All of the samples from marine sources contained a notable amount of bromophenols. Distributions of the bromophenols in the samples are listed in Tables 3.1 to 3.5 (p. 36-41). The total bromophenol contents (TBCs) of the samples are shown in Figures 3.1 to 3.6 (p. 42-47). Our results compared well with those of Boyle *et al.*'s (1992a) findings that bromophenols were widely distributed in seafood.

TBCs were used to reflect the overall flavor impact produced by each of the bromophenols. The higher the value, the stronger the flavor provided by the bromophenols, vice versa. TBCs varied widely among various seafoods. The highest bromophenol content was detected in crab meat collected in February, 2000. The TBC in this sample was 2420 *n*g/g. The lowest one was brown-spotted grouper flesh collected in August, 1999 with TBC of 2.72 *n*g/g. The TBCs also varied within a species throughout the year. All five of the bromophenols were found in 12 samples in this study, while four and three of the bromophenols were detected in

Table 3.1. Distribution of bromophenols in marine fish *Siganus canaliculatus* (Rabbitfish).

Month	Sample	Bromophenol Concentration (*n*g/g dry wt.)				
		2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]
Rabbitfish						
December, 1999	gut	30.8[b] ± 10.3[c]	206 ± 14	97.1 ± 21.2	15.3 ± 2.4	113 ± 15
	flesh	1.47 ± 0.27	ND	9.47 ± 1.05	3.52 ± 0.99	39.2 ± 4.2
February, 2000	gut	ND [d]	ND	3.41 ± 0.99	1.25 ± 0.23	24.8 ± 4.4
	flesh	ND	ND	1.97 ± 0.41	1.99 ± 0.13	18.6 ± 0.6
April, 2000	gut	0.532 ± 0.054	ND	9.09 ± 0.28	0.293 ± 0.004	18.8 ± 2.0
	flesh	1.39 ± 0.44	ND	2.82 ± 1.00	0.301 ± 0.102	15.8 ± 6.7
June, 2000	gut	7.15 ± 0.31	ND	8.14 ± 1.11	4.29 ± 0.78	9.39 ± 1.03
	flesh	0.646 ± 0.163	ND	0.788 ± 0.051	0.446 ± 0.032	6.99 ± 0.56
August, 2000	gut	1.03 ± 0.13	ND	7.11 ± 0.69	1.14 ± 0.08	3.85 ± 0.52
	flesh	0.334 ± 0.024	ND	1.22 ± 0.28	0.136 ± 0.007	4.42 ± 0.92
October, 2000	gut	4.24 ± 0.43	ND	57.0 ± 28.8	12.0 ± 0.1	129 ± 36
	flesh	0.194 ± 0.137	ND	2.14 ± 1.17	0.605 ± 0.244	20.5 ± 8.7
December, 2000	gut	7.82 ± 0.35	ND	7.62 ± 1.55	7.42 ± 1.67	26.9 ± 2.0
	flesh	10.7 ± 2.9	ND	0.727 ± 0.192	0.555 ± 0.050	27.4 ± 3.3

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol
[b]Average bromophenol concentration (*n*g/g dry wt.) from the 3 replicates
[c]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)
[d]ND = not detected

Table 3.2. Distribution of bromophenols in marine fish *Epinepheus areolatus* (Brown-spotted grouper).

Month	Sample	Bromophenol Concentration (*n*g/g dry wt.) 2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]
Brown-spotted grouper						
December, 1999	gut	8.01[b] ± 0.58[c]	ND	6.19 ± 0.11	2.28 ± 0.16	23.0 ± 1.0
	flesh	2.90 ± 0.29	ND	0.914 ± 0.052	0.835 ± 0.382	13.9 ± 2.6
February, 2000	gut	ND[d]	ND	19.1 ± 2.5	10.5 ± 3.3	107 ± 16
	flesh	ND	ND	1.33 ± 0.05	2.62 ± 0.16	15.7 ± 1.1
April, 2000	gut	3.49 ± 0.55	ND	31.4 ± 3.2	2.98 ± 0.40	155 ± 16
	flesh	ND	ND	2.97 ± 0.11	ND	19.7 ± 12.7
June, 2000	gut	15.7 ± 1.6	ND	4.63 ± 1.19	ND	2.18 ± 0.51
	flesh	3.26 ± 0.51	ND	0.578 ± 0.016	ND	2.78 ± 0.23
August, 2000	gut	2.57 ± 0.33	ND	16.5 ± 3.8	ND	24.9 ± 7.6
	flesh	ND	ND	0.286 ± 0.044	ND	2.43 ± 0.23
October, 2000	gut	17.5 ± 1.1	ND	7.08 ± 0.90	ND	30.7 ± 3.3
	flesh	4.18 ± 0.38	ND	ND	ND	21.8 ± 0.7
December, 2000	gut	8.28 ± 0.54	ND	6.78 ± 0.47	1.68 ± 0.14	19.4 ± 1.4
	flesh	1.31 ± 0.61	ND	5.36 ± 0.07	0.598 ± 0.342	8.51 ± 0.33

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol
[b]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates
[c]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)
[d]ND = not detected

Table 3.3. Distribution of bromophenols in the mollusk *Tape philippinarum* (Clam).

Month	Bromophenol Concentration (*n*g/g dry wt.)				
	2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]
Clam					
December, 1999	17.2[b] ± 0.1[c]	ND [d]	46.7 ± 0.5	8.99 ± 0.80	85.9 ± 7.4
February, 2000	14.1 ± 1.1	55.6 ± 8.9	44.5 ± 9.9	4.72 ± 0.23	93.7 ± 10.3
April, 2000	0.170 ± 0.003	ND	2.54 ± 0.26	0.289 ± 0.023	7.28 ± 0.67
June, 2000	1.85 ± 0.09	ND	39.5 ± 3.5	0.146 ± 0.016	11.1 ± 0.8
August, 2000	4.32 ± 0.20	ND	195 ± 16	2.12 ± 0.22	43.2 ± 3.5
October, 2000	5.99 ± 0.41	17.5 ± 0.9	64.6 ± 6.8	1.99 ± 0.14	43.4 ± 3.1
December, 2000	4.34 ± 0.11	4.62 ± 0.35	27.4 ± 2.8	11.9 ± 1.2	198 ± 15

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol
[b]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates
[c]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)
[d]ND = not detected

Table 3.4. Distribution of bromophenols in the mollusk *Ostrea rivularis* (Oyster).

Month	Bromophenol Concentration (*n*g/g dry wt.)				
	2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]
Oyster					
December, 1999	NA	NA [e]	NA	NA	NA
February, 2000	6.10[b] ± 0.26[c]	ND [d]	51.6 ± 0.5	0.667 ± 0.014	13.3 ± 0.5
April, 2000	2.54 ± 0.58	ND	52.9 ± 11.5	2.18 ± 0.48	18.1 ± 3.0
June, 2000	2.07 ± 0.15	ND	9.80 ± 1.13	0.345 ± 0.038	1.37 ± 0.15
August, 2000	1.85 ± 0.16	ND	11.0 ± 1.6	0.815 ± 0.086	3.30 ± 0.27
October, 2000	2.58 ± 0.34	ND	17.3 ± 0.9	0.395 ± 0.024	2.33 ± 0.55
December, 2000	3.96 ± 0.44	ND	41.2 ± 7.9	0.744 ± 0.021	15.5 ± 0.4

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol
[b]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates
[c]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)
[d]ND = not detected
[e]NA = sample not available

Table 3.5. Distribution of bromophenols in crustacean *Penaeus japonicus* (Shrimp).

Month	Sample	Bromophenol Concentration (*n*g/g dry wt.)				
		2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]
Shrimp						
December, 1999	head	3.85[b] ± 0.48[c]	ND [d]	9.39 ± 1.33	14.4 ± 1.4	154 ± 13
	body	3.62 ± 0.46	ND	5.57 ± 0.84	6.09 ± 0.40	12.3 ± 2.0
February, 2000	head	5.70 ± 1.53	ND	4.90 ± 2.45	1.13 ± 0.15	77.7 ± 26.4
	body	0.421 ± 0.029	ND	0.613 ± 0.153	0.319 ± 0.066	17.4 ± 3.0
April, 2000	head	3.84 ± 0.14	ND	9.64 ± 1.00	3.34 ± 0.24	49.5 ± 6.4
	body	0.617 ± 0.129	ND	3.80 ± 0.52	0.926 ± 0.070	10.2 ± 0.4
June, 2000	head	1.27 ± 0.21	ND	1.78 ± 0.18	0.431 ± 0.068	8.30 ± 0.35
	body	0.446 ± 0.066	ND	1.08 ± 0.08	0.824 ± 0.061	7.32 ± 0.08
August, 2000	head	7.72 ± 0.86	3.34 ± 0.09	1.81 ± 0.02	0.818 ± 0.048	7.65 ± 0.21
	body	2.71 ± 0.28	ND	6.78 ± 4.97	ND	6.38 ± 0.52
October, 2000	head	6.26 ± 0.57	ND	5.98 ± 0.18	5.35 ± 0.26	40.8 ± 30.0
	body	5.87 ± 0.73	ND	ND	0.465 ± 0.037	12.9 ± 5.3
December, 2000	head	1.53 ± 0.04	9.47 ± 0.15	10.4 ± 0.7	3.85 ± 0.20	62.0 ± 6.5
	body	0.841 ± 0.106	5.39 ± 0.39	2.64 ± 0.32	2.68 ± 0.42	15.8 ± 0.6

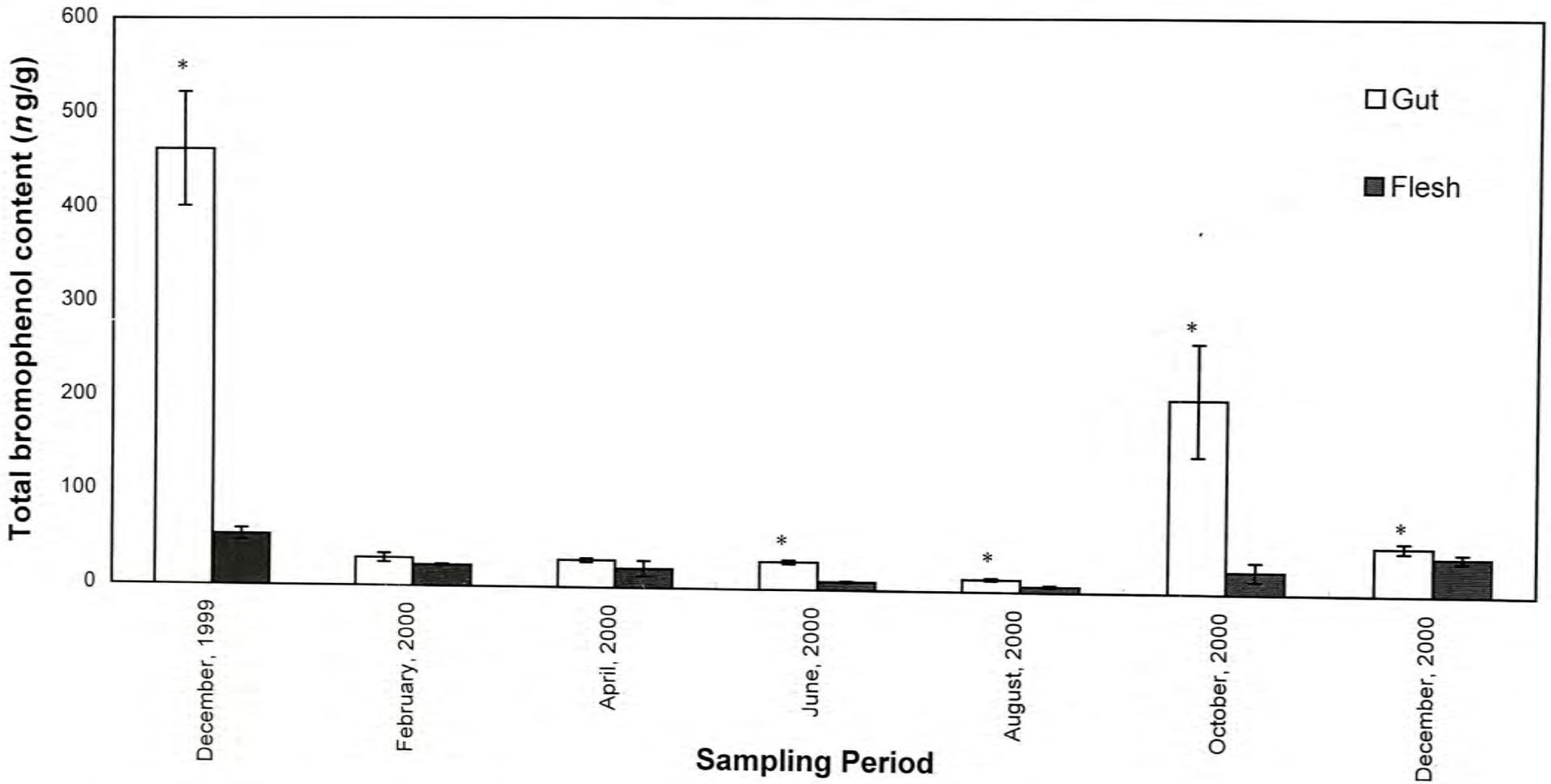
[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol
[b]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates
[c]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)
[d]ND = not detected

Table 3.6. Distribution of bromophenols in crustacean *Charybdis feriatus* (Crab).

Month	Bromophenol Concentration (*n*g/g dry wt.)				
	2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]
Crab					
December, 1999	2.94[b] ± 0.16[c]	1.74 ± 0.04	54.2 ± 4.8	2.31 ± 0.14	336 ± 28
February, 2000	5.11 ± 0.12	ND [d]	43.8 ± 5.2	5.47 ± 0.64	2360 ± 91
April, 2000	34.4 ± 1.8	3.88 ± 0.13	68.1 ± 0.5	77.3 ± 11.2	295 ± 11
June, 2000	0.991 ± 0.060	ND	53.0 ± 2.8	6.29 ± 0.25	38.6 ± 2.1
August, 2000	2.76 ± 0.24	4.16 ± 0.10	27.6 ± 1.1	8.63 ± 0.93	151 ± 22
October, 2000	3.40 ± 0.09	22.6 ± 2.5	32.0 ± 0.4	15.0 ± 1.3	132 ± 2
December, 2000	8.54 ± 0.69	47.9 ± 4.6	214 ± 20	7.81 ± 0.98	501 ± 57

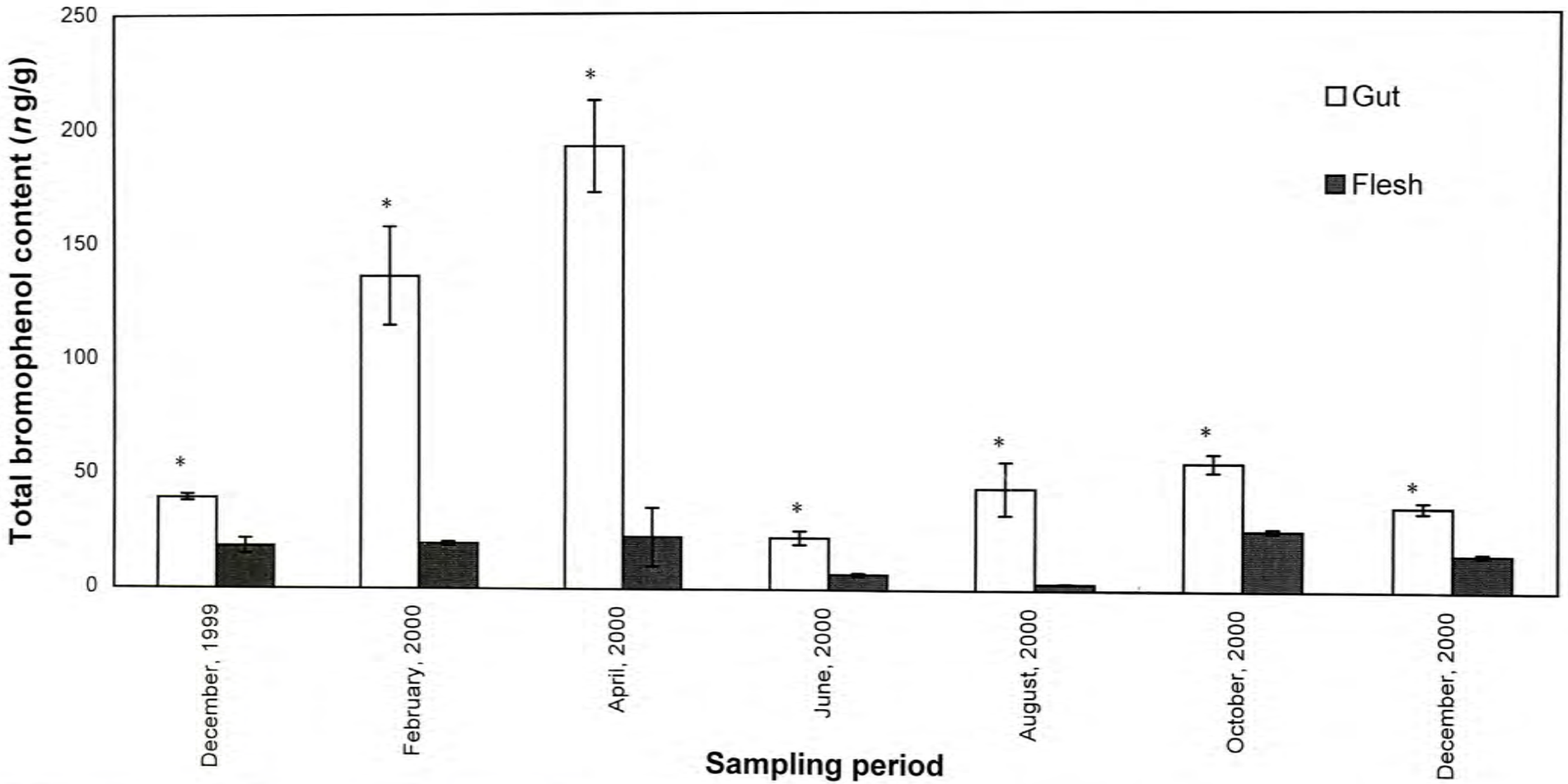
[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol
[b]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates
[c]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)
[d]ND = not detected

Figure 3.1. Mean (±SD) total bromophenol content in the gut and the flesh of rabbitfish (*Siganus canaliculatus*) over time (n=3).



*The total bromophenol content in gut was significantly higher than that in flesh at the same period compared by t-test ($p<0.05$).

Figure 3.2. Mean (±SD) total bromophenol content in the gut and the flesh of brown-spotted grouper (*Epinephelus areolatus*) over time (n=3).



*The total bromophenol content in gut was significantly higher than that in flesh at the same period compared by t-test ($p<0.05$).

Figure 3.3. Mean (±SD) total bromophenol content of clam (*Tape philippinarum*) over time (n=3).

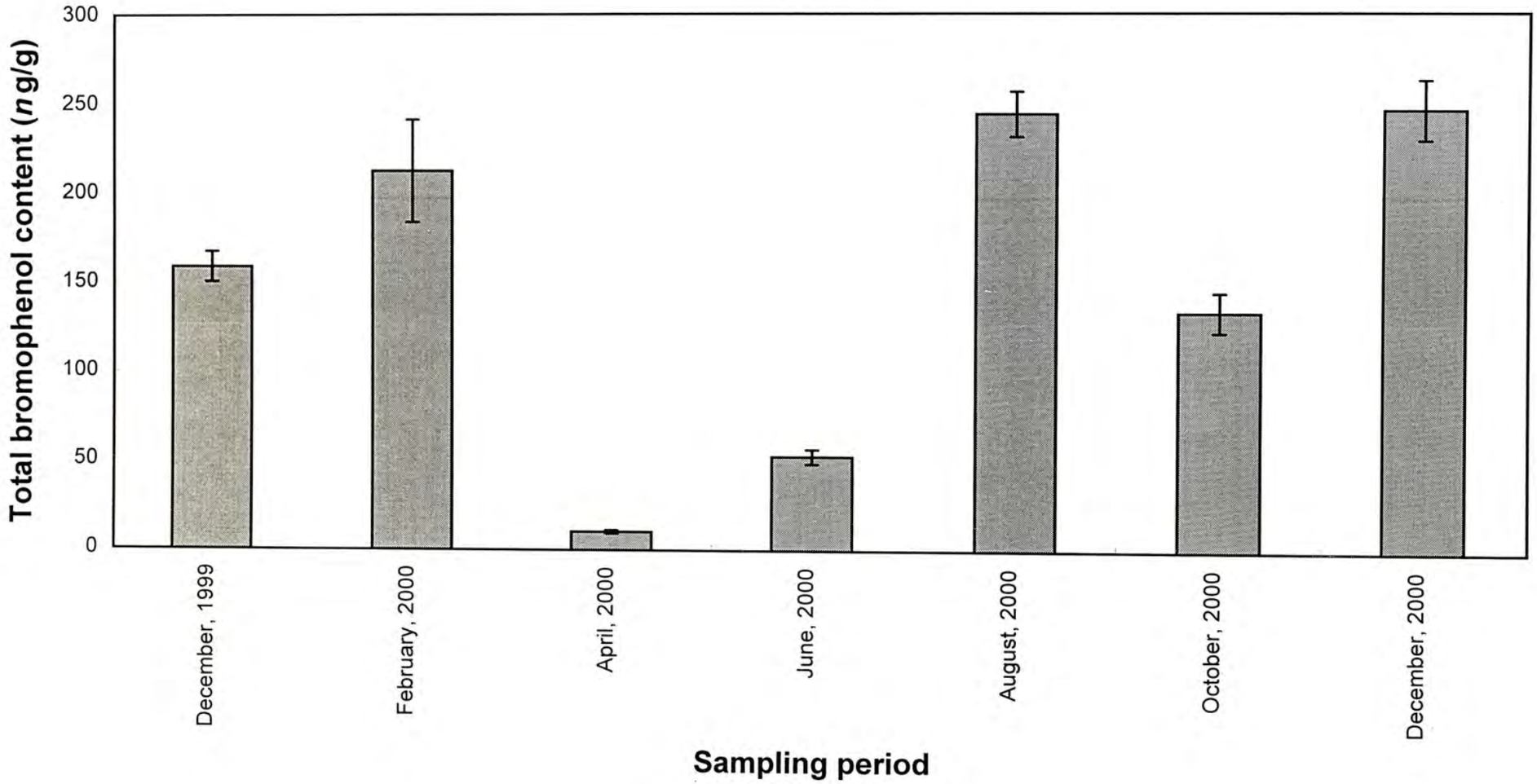
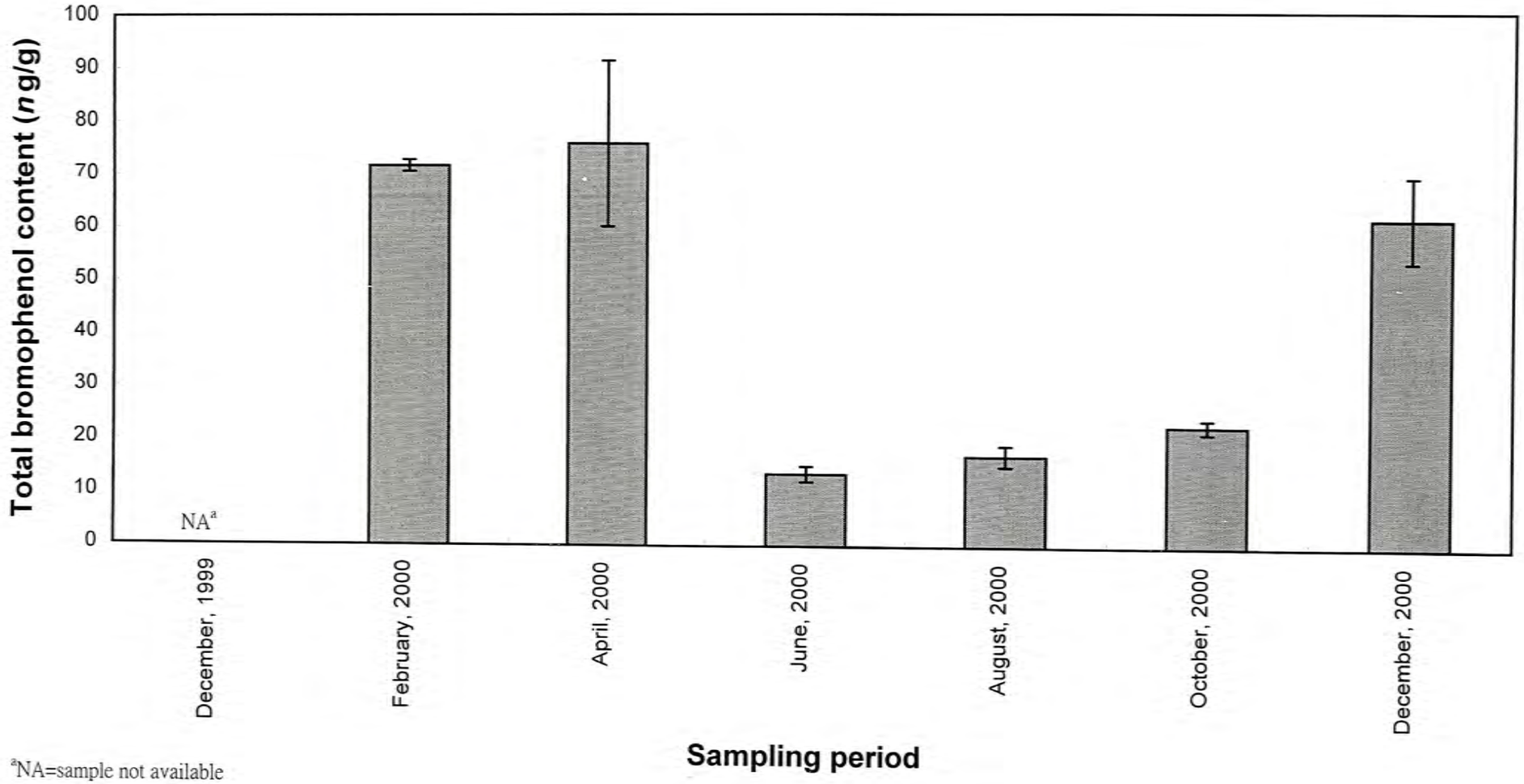
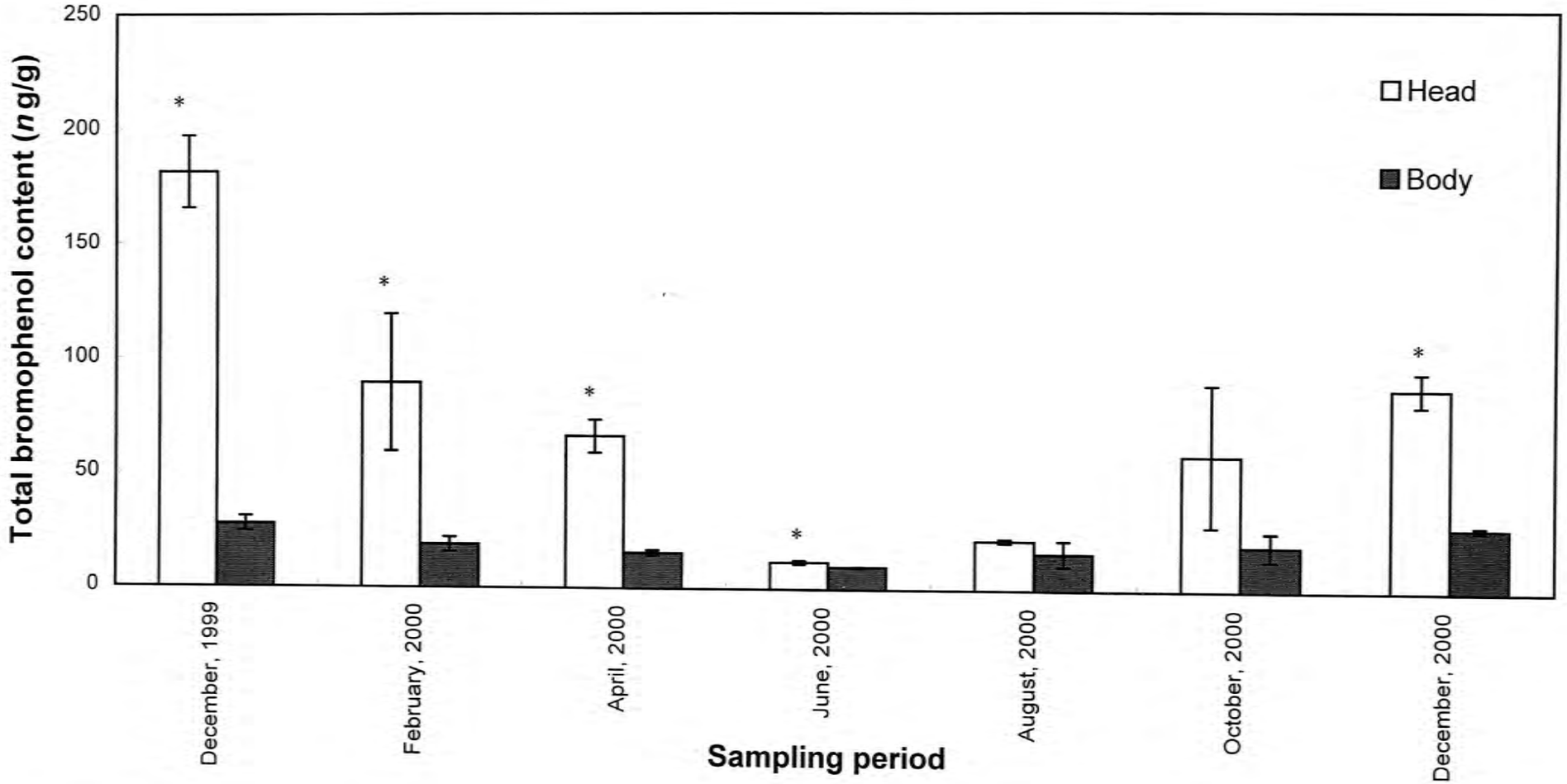


Figure 3.4. Mean (±SD) total bromophenol content of oyster (*Ostrea rivularis*) over time (n=3).



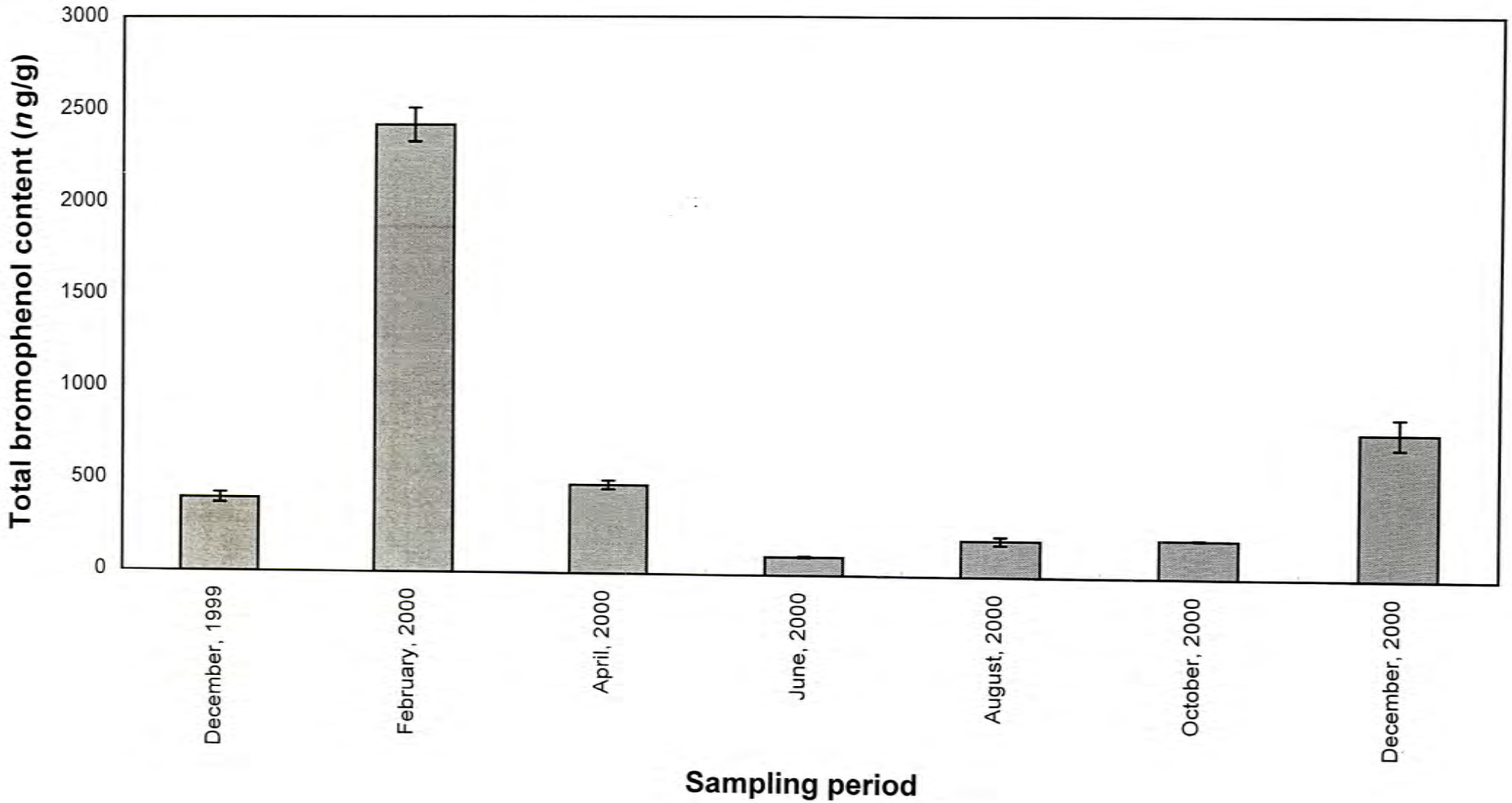
[a]NA=sample not available

Figure 3.5. Mean (±SD) total bromophenol content in the head and the body of shrimp (*Penaeus japonicus*) over time (n=3).



*The total bromophenol content in head was significantly higher than that in body at the same period compared by t-test ($p<0.05$).

Figure 3.6. Mean (±SD) total bromophenol content in the meat of crab (*Charybdis feriatus*) over time (n=3).



37 and 10 samples, respectively.

Among the five bromophenols studied, 2,4,6-tribromophenol was the most abundant one and was detected in all seafood samples. 2,4-Dibromophenol, 2-bromophenol and 2,6-dibromophenol were found only in 96.8%, 90.3% and 87.1% of the samples, respectively. The least abundant one was 4-bromophenol which appeared in only 19.4% of the samples. Apart from the frequency of detection, 2,4,6-tribromophenol contributed quantitatively the largest proportion among the bromophenols in 46 samples (74.2%). In 12 samples (19.4%), 2,4-dibromophenol possessed the highest concentration. 2-BP and 4-BP concentrations were the highest in 3 and 1 samples, respectively.

The presence of each individual bromophenol varied greatly among species. For the most abundant 2,4,6-TBP, the concentrations detected ranged from 2.18 *n*g/g in brown spotted grouper (*Epinephelus areolatus*) gut in June, 2000 to 2360 *n*g/g in crab (*Charybdis feriatus*) in February, 2000. For 2-BP, 4-BP, 2,4-BP and 2,6-BP, the concentrations detected among the samples varied from 0 *n*g/g [i.e. not detected (ND)] to 34.4 *n*g/g, 206 *n*g/g, 214 *n*g/g and 77.3 *n*g/g, respectively. The variations were extremely high. Besides, the distributions of the bromophenols in each

species collected at different period also fluctuated greatly.

3.3.1.1 Bromophenols in marine fishes

Two kinds of marine fishes, rabbitfish (*Siganus canaliculatus*) and brown-spotted grouper (*Epinephelus areolatus*), which are commonly consumed in Hong Kong, were studied in this survey. The bromophenol content in gut and in flesh were analyzed separately. Bromophenols were detected in both gut (representing the dietary intake) and flesh in all samples. In the gut, the TBC detected in rabbitfish ranged from 13.1 to 462 *n*g/g, and in brown-spotted grouper, from 22.5 to 193 *n*g/g. Considering the flesh, the TBCs in rabbitfish ranged from 6.12 to 53.7 *n*g/g whereas in brown-spotted grouper ranged from 2.72 to 26.0 *n*g/g. These ranges showed that the TBCs varied more widely in gut than in the flesh. In all of the 14 samples examined, the TBCs in guts were higher than those in the flesh. The most abundant bromophenol was 2,4,6-TBP which could be found in all samples, while 4-BP could be detected in only one of the gut samples.

3.3.1.2 Bromophenols in mollusks

The bromophenol contents in clam (*Tape philippinarum*) and oyster (*Ostrea rivularis*) were investigated. The TBCs in mollusks were relatively high when

compared with those in other species. Bromophenols were detected in all of the samples ranging from 10.3 to 246 *n*g/g in clam and 13.6 to 75.8 *n*g/g in oyster. In August, 2000, the TBC in clam was the highest among the samples examined in the same period. Among the 5 bromophenols, 2-BP, 2,4-DBP, 2,6-DBP and 2,4,6-TBP appeared in all 13 samples. Both 2,4-DBP and 2,4,6-TBP were more abundant in clam than the other bromophenols. But for oyster, only 2,4-DBP was the most abundant one, an observation which was different from other seafood samples.

3.3.1.3 Bromophenols in crustaceans

Two types of crustaceans, shrimp (*Penaeus japonicus*) and crab (*Charybdis feriatus*) were selected. 2,4,6-TBP was found to be the most abundant among the bromophenols. In shrimp, the bromophenol contents in the head (the cephalothorax part) and the body (abdomen) were analyzed separately. The TBCs in the shrimp heads (11.8 to 181 *n*g/g) were higher than those in the bodies (9.67 to 27.5 *n*g/g). In crab, the TBCs detected were generally the highest among all the seafood samples in the same month except in December, 1999 and August, 2000. The TBC was highest in Feb, 2000 (2410 *n*g/g) and lowest in June, 2000 (98.8 *n*g/g). All bromophenols except 4-BP could be detected in the crabs throughout the study. 4-BP was found in five samples. According to Boyle *et al.* (1992a), crabs contained

an abundant amount of bromophenols that could contribute to the crab flavor. The high concentrations of bromophenols appeared to be related to the distinct briny flavors of crabmeat.

3.3.2 Seasonal variations of the TBCs

The seasonal variations of the TBCs of different samples throughout the year are shown in Figures 3.1 to 3.6 (p. 42 - 47). Among all the samples, the TBCs detected varied widely. The lowest TBC detected was 2.72 ng/g in brown-spotted grouper flesh analyzed in August, 1999. The highest TBC detected was 2420 ng/g in crab meat analyzed in February, 2000.

In both marine fishes, rabbitfish and brown-spotted grouper, the TBCs did not fluctuate widely in the flesh, but did so in the gut [Figures 3.1 and 3.2 (p. 42 - 43)]. In clam and oyster, the TBCs fluctuated [Figures 3.3 and 3.4 (p.44 - 45)]. Besides, the TBCs in shrimp body remained relatively constant when compared with that of the head [Figure 3.5 (p.46)]. The TBCs in crab varied greatly at high concentrations [Figure 3.6 (p. 47)].

Generally, the TBCs in different kinds of seafoods were higher in those seasons

with lower temperatures (i.e. from October to April). The TBCs were lower in summer (June and August). For the clam, there was a sudden increase in the concentration of bromophenol in August, 2000. As bromophenols were thought to be accumulated from diets (Boyle *et al.*, 1992a), the cause of the variations of the TBCs might be due to sudden changes in the diet compositions containing different concentrations of bromophenols.

3.3.3 Bromophenols in diet contents

In this study, the gut of the fishes and the head (with gut) of the shrimp were separated from their flesh and body, and bromophenols were extracted and analyzed separately. The bromophenol contents of the gut and the head section of the samples represented the bromophenol contents of their diet contents (Whitfield *et al.*, 1997b). For those bromophenols found in the flesh of a fish sample, they were also found in its gut sample. Similar condition was found between the body and head of shrimp. Previous study on Australian prawns also showed such relationship (Whitfield *et al.*, 1997b). In all the samples, the TBCs in the gut/head were always higher than those of the flesh/body. In five samples of rabbit fish, seven samples of brown-spotted grouper and five samples of shrimp, their TBCs in gut/head were significantly higher than that in the flesh/body [Figures 3.1, 3.2 and 3.5 (p. 42, 43

and 46)] compared by t-test at $p<0.05$. The TBC ratio of gut and flesh or head and body was calculated for each sample [Table 3.7 (p. 54)]. All of the ratios were higher than one, e.g. 1.26 – 8.65 in rabbit fish, 2.12 - 16.2 in brown-spotted grouper and 1.22 – 6.59 in shrimp. Their averages were 3.81, 5.94 and 3.49, respectively. In the study of Australian prawns (Whitfield *et al.*, 1997b), the average ratio was 7.7 which was higher than that of the present study. Previous studies on marine fishes and prawns suggested that bromophenols were accumulated in the animal's natural diets (Whitfield *et al.*, 1997b; 1998). The higher bromophenol concentrations in gut/head than in flesh/body strongly support this view because the TBCs in the diet contents were relatively high. The higher average TBC ratio of head and body in Australia would be explained by higher bromophenol contents in their diets than those in Hong Kong.

3.3.4 Bromophenol contents of freshwater fish

None of the bromophenols was detected in the freshwater fish sample *Ctenopharyngodon idellus* (grass carp) [Table 3.8 (p.55)]. It is a pond fish in Hong Kong feeding on higher plants and detritus (Man and Hodgkiss, 1981). In 1992, Boyle *et al.* (1992a) investigated the bromophenol content in freshwater fish, including Cisco herring, whitefish, rainbow trout, northern pike and walleye pike.

Table 3.7. Ratios of mean total bromophenol content in gut and flesh or head and body in selected samples.

	Siganus canaliculatus (Rabbit fish) Gut/Flesh	*Epinephelus areolatus* (Brown-spotted grouper) Gut/Flesh	*Penaeus japonicus* (Shrimp) Head/Body
December, 1999	8.61	2.13	6.59
February, 2000	1.31	6.94	4.76
April, 2000	1.41	8.50	4.26
June, 2000	3.27	3.40	1.22
August, 2000	2.15	16.2	1.34
October, 2000	8.65	2.12	3.04
December, 2000	1.26	2.29	3.19
Average	3.81±3.37	5.94±5.19	3.49±1.91

Table 3.8. Distribution of bromophenols in a freshwater fish *Ctenopharyngodon idellus* (Grass Carp).

Month	Bromophenol Concentration (*n*g/g dry wt.)				
	2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]
Grass Carp					
December, 1999	ND [b]	ND	ND	ND	ND

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol

[b]ND = not detected

In these samples, bromophenols were virtually absent. This phenomenon could be explained by the lack of bromine and the flora or fauna responsible for the biosynthesis of bromophenols (Fenical, 1981).

3.3.5 Relationship between the living habitats and bromophenol contents

As the bromophenols present in the marine animals originated from their diets (Whitfield *et al.*, 1998), the habitats and their feeding habits directly affected their bromophenol contents. It was possible to relate the living habitats of the animals to the amount of bromophenols they possessed. For example, marine rabbitfish, which dwell in algal or seabed, feed mainly on marine algae containing high amounts of bromophenols (Whitfield *et al.*, 1999b; Lau and Li, 2000). Brown-spotted grouper (*Epinephelus areolatus*) commonly found in seagrass beds or fine sediment bottom (Lau and Li, 2000) feeds mainly on crustaceans. Since crustaceans are incapable of producing bromophenols, the bromophenols obtained in grouper that feeds on crustaceans were on a secondary level along the food web. This might explain the observation that bromophenol contents in rabbitfish (TBCs: 6.12 – 53.7 *n*g/g) were higher than that in brown-spotted grouper (TBCs: 2.72 – 26.0 *n*g/g).

Mollusks such as clam and oyster are filter feeders (Orr, 1985; Shumway *et al.*,

1986). Clam, which lives in planktonic and benthic habitats at different developmental stages (Carriker, 1986), relies on a variety of food sources. Similarly, oyster is commonly found on rocks. They feed on the organic substrate (Lotsy, 1895; Grave, 1916) and organisms suspended in water. Mollusks could obtain bromophenols from bromophenol-producing plankton or algal detritus (Boyle *et al.*, 1993; Whitfield *et al.*, 1999b). High concentrations of 2,4-DBP detected in oyster might imply their habitat containing food sources with high concentration of 2,4-DBP.

Shrimp and crab are both benthic animals. Shrimp (*Penaeus spp.*) was omnivorous feeding on a variety of phytoplankton and other benthic animals such as crustaceans and polycheates (Villaluz *et al.*, 1969; Pascual, 1988), which contained high concentrations of bromophenols (Whitfield *et al.*, 1999a). Bromophenols could be obtained from such sources. Crab (*Charybdis feriatus*) is a carnivorous animal. Their food sources are mainly benthic animals such as bivalves, gastropods and polychaetes (Warner, 1977). The extremely high concentrations of bromophenols in crab found in this study (highest concentration at 2420 *n*g/g) might be due to their consumption of large amount of bromophenol-rich polychaetes. Besides, bromophenol accumulation in crab might be higher in those uncommon

organs such as carapace. Overall, the bromophenol contents in the marine animals have a close relationship with the animals' habitats.

3.3.6 Bromophenols as flavor compounds in seafoods

Bromophenols were a group of key flavor compounds in seafood with low threshold values (Whitfield *et al.*, 1988). The threshold values are shown in Table 2.2 (p. 16). To produce the desirable marine-like or sea-like flavor, at least one of the bromophenols, 2-BP, 2,6-DBP and 2,4,6-DBP, should exceed the concentrations of 10 *n*g/g, 0.1 *n*g/g and 10 *n*g/g, respectively (Boyle *et al.*, 1992b). The concentrations of bromophenols in the seafood, especially clam and crab, found in Hong Kong market were sufficient to produce the desirable brine- or sea-like flavors (Boyle *et al.*, 1992a; b; Whitfield *et al.*, 1988). In fact, their amounts were generally higher than the threshold values found in the literature (Whitfield *et al.*, 1988) that might affect their general flavor.

The most potent bromophenol, 2,6-DBP, which was the cause of iodine-like off-flavor in prawns, was detected in 87.1% of the samples. However, the amount present did not cause iodine-like off-flavor in the seafoods studied as the concentrations detected were generally low (0.289 – 77.3 *n*g/g dry weight). The

concentration required to cause an off-flavor in prawn meat was 32 ng/g on a wet weight basis (Whitfield *et al.*, 1988). In the samples of the present study, the highest concentration of 2,6-DBP detected in crabmeat (April, 2000) was 77.3 *n*g/g on a dry weight basis. The actual concentration would be lower on a wet weight basis. The recalculated value is 14.4 *n*g/g at 80% moisture. Therefore, seafoods in the present study would likely be free from the iodine-like off-flavor.

3.4 Conclusion

Bromophenols were widely distributed in selected Hong Kong seafood of marine origin. The bromophenol contents varied among species and within samples at different periods. Generally, the concentrations of the bromophenols in most of the seafoods were sufficient to contribute the distinct sea-like or brine-like flavor. 2,4,6-TBP was the most abundant one among all samples chosen.

The TBC in gut was much higher than that in the meat. This strongly supports the view that bromophenols were obtained from the diets of the animals. The results also showed that bromophenols were detected in seafoods from marine sources but not detected in the freshwater sample. Such phenomenon can be explained by the differences in the diets they consumed. Dietary sources of

bromophenols are available in seawater environment but not freshwater one.

This study provide valuable information on the quantitative presence of bromophenols in seafoods commonly found in Hong Kong. Further studies should focus on isolating some possible sources of bromophenols in the marine environment.

Chapter 4

Distributions of Bromophenols in selected Hong Kong seaweeds

4.1 Introduction

The occurrence of bromophenols in seafood has been extensively investigated (Boyle *et al.*, 1992a; Whitfield *et al.*, 1997b; 1998). Bromophenols were commonly encountered in seafoods from different marine sources and they were responsible for the sea-like and brine-like flavor (Boyle, 1992a; b). In Hong Kong, the distributions of the five simple bromophenols including 2-bromophenol (2-BP), 4-bromophenol (4-BP), 2,4-dibromophenol (2,4-DBP), 2,6-dibroophenol (2,6-DBP) and 2,4,6-tribromophenol (2,4,6-TBP) in several seafoods during different seasons were analyzed and discussed in the previous chapter (chapter 3). Based on the previous studies, it is strongly believed that bromophenols present in seafood were obtained from nature. It will be necessary to locate some dietary sources containing abundant amount of these compounds. Studies on the bromophenol content in Australian marine algae showed that there was a significant amount of bromophenols detected that may contribute to the flavor of fish (Whitfield *et al.*, 1998; 1999b). In herbivorous fish, marine algae were the major sources of these bromine-containing compounds (Whitfield *et al.*, 1998). The presence of bromophenols in algae was

due to the possession of bromoperoxidases which are the enzymes responsible for brominating the organic substrates in the presence of bromide ion and hydrogen peroxide (Flodin and Whitfield, 1999b). In the marine environment surrounding Hong Kong, it is also true that marine algae are one of the main dietary sources of many marine organisms. However, information on their role as sources of bromophenols is not available.

In the current study, the bromophenol contents of selected marine algae and the seasonal variations of such compounds were monitored.

4.2 Materials and methods

4.2.1 Sample collection and preparation

Three species of marine algae including *Padina arborescens*, *Sargassum siliquastrum* and *Lobophora variegata* were freshly collected from Tung Ping Chau, Hong Kong SAR, China every two months from December, 1999 to October, 2000. Samples were transported to the laboratory immediately. They were gently washed with double distilled water and excess water was drained out. The samples were packed in plastic bags (23 cm × 30 cm) and stored at -80°C until extractions were carried out.

Organic solvents pentane and diethyl ether were purchased from Lab-scan Ltd. (Ireland) with purity of 99% and 99.5% respectively. Standard samples of 2-BP, 2,4-DBP and 2,6-DBP were bought from Aldrich Chemical Co. (Milwaukee, WI), and 4-BP and 2,4,6-TBP were purchased from Acros Organics (Belgium). The purities of these five bromophenols ranged from 97% to 99%.

4.2.2 Simultaneous steam distillation-solvent extraction (SDE)

The extraction method was adapted from Whitfield *et al.* (1988, 1997b). Each sample was homogenized by a National Blender (MX-T2GM, Matsushita Electric Co. Ltd., Taipei, Taiwan) for four minutes. Treated samples, 1 mL of internal standard (1,3,5-trimethylbenzene, concentration at 84.7 μg/mL) and 500 mL of boiled double distilled water were transferred to a 5-L round bottom flask. The sample was then acidified to pH 1 with 96% sulphuric acid. Extraction with 40-mL of pentane/diethyl ether (9:1 v/v) for 2.5 hours in a Likens and Nickerson type SDE apparatus (Cat. No. K-523010-0000, Kontes, Vineland, NJ) was then carried out. The pH of the residue after extraction was measured again to ensure that the acidity was maintained throughout the process. Triplicate extractions of each sample were carried out. Extract collected was further concentrated to 0.25 mL with a stream of

ultra high purity (99.999%) nitrogen and dried over 2.85 g anhydrous sodium sulfate. The concentrated extract was temporary stored in a 15-mL conical tube at –80°C until further analyses were carried out.

4.2.3 Gas chromatography/mass spectrometry (GC/MS)

A GC/MS system consisting of a Hewlett-Packard 6890 GC coupled with a HP 5973 mass selective detector (MSD) (Hewlett-Packard Co., Palo Alto, CA) was used for qualitative and quantitative analyses. Five μL of each extract was injected, in splitless mode with injector temperature at 200°C, into a fused silica open tubular column (Supelcowax-10, 60 m length × 0.25 mm i.d. × 0.25 μm film thickness; Supelco, Inc., Bellefonte, PA). Helium gas (ultra high purity grade, 99.999%) was used as carrier gas at constant linear velocity at 30 cm/s. Oven temperature was programmed from 100 to 200°C at a ramp rate of 10°C/min. The initial and final hold times were 5 and 75 mins., respectively. MS interface, ion source and MS quadrupole temperatures were set at 250°C, 230°C and 106°C, respectively. Ionization voltage was 70 eV and electron multiplier voltage was 1200 V. Selected ion monitoring (SIM) GC/MS procedure was used. Ions were monitored for 2- and 4-bromophenol (2- and 4-BP) at *m/z* 172 and 174; for 2,4- and 2,6-dibromophenol (2,4- and 2,6-DBP) at *m/z* 250 and 252; for 2,4,6-tribromophenol

(2,4,6-TBP) at *m/z* 330 and 332; and for internal standard 1,3,5-trimethylbenzene at *m/z* 105 and 120 (Lee *et al.*, 1984; Whitfield *et al.*, 1997b; Chung, 1999).

4.2.4 Compound identification and quantification

The presence of each bromophenol was confirmed by the detection of a single peak in the selected ion chromatogram at corresponding retention time and by the presence of the two characteristic ions listed above with particular isotopes ratios (Whitfield *et al.*, 1997b).

For quantification, 3-point standard curves for each bromophenol were established. Solutions (5mL) containing 5 mg of each of the five bromophenols was prepared. Serial dilutions at ratios of 1:5 and 1:25 were made. A constant amount of internal standard, 1,3,5-trimethylbenzene (5 mg), was added into each of the above solutions prepared. The area ratios from both selected ions (bromophenol/internal standard) were plotted against the amount ratios to obtain a response factor for each bromophenol. Ions chosen were with *m/z* of 172 (monobromophenols), 252 (dibromophenols), 330 (tribromophenol) and 105 (internal standard). When response factor of each compound was obtained, the amount ratios (bromophenol/internal standard) of each bromophenol in the extracts

could be calculated. Concentration of a bromophenol in a sample was calculated by the following equation (Equation 1):

Concentration of a bromophenol (*n*g/g dry weight)

$$= \frac{\text{(amount ratio of bromophenol/internal standard)} \times \text{amount of internal standard } (n\text{g})}{\text{dry weight of sample (g)}}$$

.....Equation 1

The total bromophenol content (TBC) was calculated by the following equation (Equation 2):

Total bromophenol content (TBC) (*n*g/g dry weight)

=Summation of the concentrations of 2-BP, 4-BP, 2,4-DBP, 2,6-DBP and 2,4,6-TBP (*n*g/g)

.....Equation 2

4.2.5 Recoveries

The recoveries of the SDE technique on the various bromophenols were calculated according to Equation 3. Briefly, it was determined by extracting known amount of each bromophenol with the SDE method, and quantified with the same GC/MS system under the same experimental conditions described above. These values were used to calculate the original amount of bromophenols in the samples. The average recoveries of the bromophenols were listed in chapter 3 (p.35).

Recovery (%)

$$= \frac{\text{Concentration of bromophenol detected}}{\text{Concentration of bromophenol standard added}} \times 100\% \qquad \text{.....Equation 3}$$

4.2.6 Moisture determination

The percentage moisture of each sample was determined according to the instructions in the operation manual of the Mettler LJ16 moisture analyzer (Mettler-Toledo, Switzerland). Dry weights of the samples were then determined to calculate the concentration of each bromophenols in seaweeds.

4.3 Results and discussions

4.3.1 Distribution of bromophenols in marine algae

The distributions of the five targeted bromophenols in three species of marine algae commonly found in Hong Kong water were revealed. Every 2 months from December, 1999 to October, 2000, seaweeds were collected and bromophenols were determined to obtain their seasonal variations of such compounds. Results are shown in Table 4.1 - 4.3 (p.68-70) and Figures 4.1 - 4.3 (p.71-73).

Three species of macroalgae, *Padina arborescens*, *Sargassum siliquastrum* and

Table 4.1. Distribution of Bromophenols in the marine algae *Padina arborescens.*

Species / Month	Bromophenol Concentration (*n*g/g dry wt.)				
	2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]
Padina arborescens					
December, 1999	6.38[b] ± 0.90[c]	25.3 ± 2.6	347 ± 40	10.3 ± 0.68	929 ± 11
February, 2000	1.31 ± 0.22	50.5 ± 24.0	116 ± 33	24.3 ± 10.5	547 ± 38
April, 2000	59.8 ± 2.9	56.7 ± 3.6	357 ± 20	54.6 ± 3.0	602 ± 54
June, 2000	2.33 ± 0.10	9.72 ± 0.86	12.8 ± 3.3	1.54 ± 0.17	14.6 ± 0.9
August, 2000	NA [d]	NA	NA	NA	NA
October, 2000	44.6 ± 10.0	95.7 ± 14.2	102 ± 7	35.0 ± 7.8	246 ± 37

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol
[b]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates
[c]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)
[d]NA=sample not available due to dying back

Table 4.2. Distribution of Bromophenols in the marine algae *Sargassum siliquastrum.*

Species / Month	Bromophenol Concentration (*n*g/g dry wt.)				
	2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]
Sargassum siliquastrum					
December, 1999	4.36[b] ± 1.02[c]	1260 ± 30	120 ± 14	15.6 ± 0.8	2430 ± 50
February, 2000	0.765 ± 0.051	45.0 ± 4.4	550 ± 32	110 ± 17	1790 ± 210
April, 2000	22.9 ± 1.9	109 ± 18	831 ± 134	56.6 ± 8.1	1060 ± 220
June, 2000	NA [d]	NA	NA	NA	NA
August, 2000	1.18 ± 0.13	45.9 ± 8.5	45.8 ± 5.9	48.0 ± 1.8	345 ± 84
October, 2000	13.9 ± 0.5	45.3 ± 3.4	265 ± 15	14.6 ± 2.2	2270 ± 130

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol
[b]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates
[c]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)
[d]NA=sample not available due to dying back

Table 4.3. Distribution of Bromophenols in the marine algae *Lobophora variegata.*

Species / Month	Bromophenol Concentration (*n*g/g dry wt.)				
	2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]
Lobophora variegata					
December, 1999	ND [e]	73.0[b] ± 19.6[c]	1070 ± 90	23.6 ± 1.3	5870 ± 460
February, 2000	ND	244 ± 33	461 ± 141	45.4 ± 4.3	1600 ± 400
April, 2000	ND	165 ± 27	340 ± 37	78.8 ± 5.1	975 ± 60
June, 2000	NA [d]	NA	NA	NA	NA
August, 2000	ND	527 ± 105	736 ± 97	73.5 ± 12.5	1190 ± 150
October, 2000	ND	243 ± 14	1280 ± 100	37.4 ± 0.9	1070 ± 130

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol

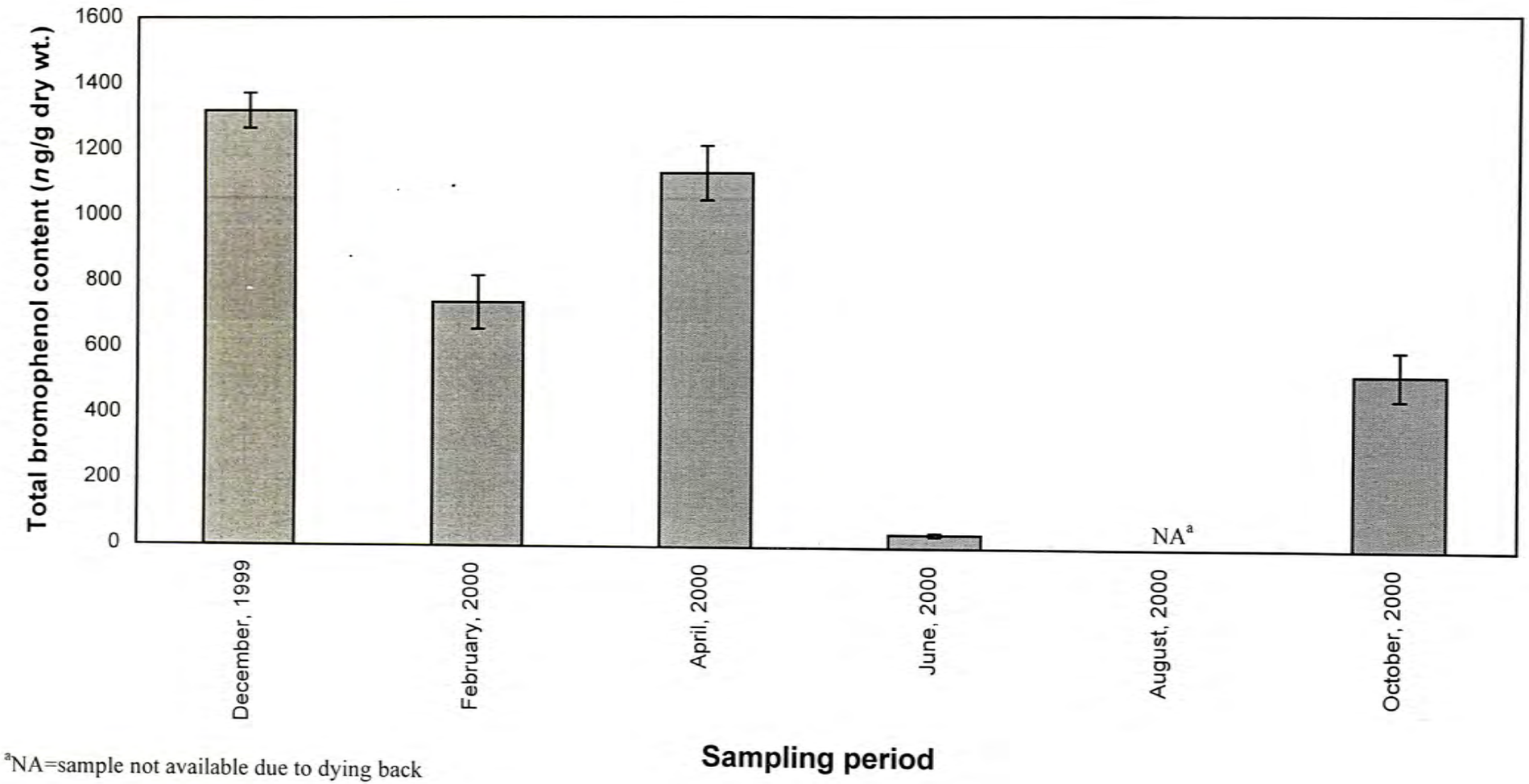
[b]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates

[c]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)

[d]NA=sample not available due to dying back

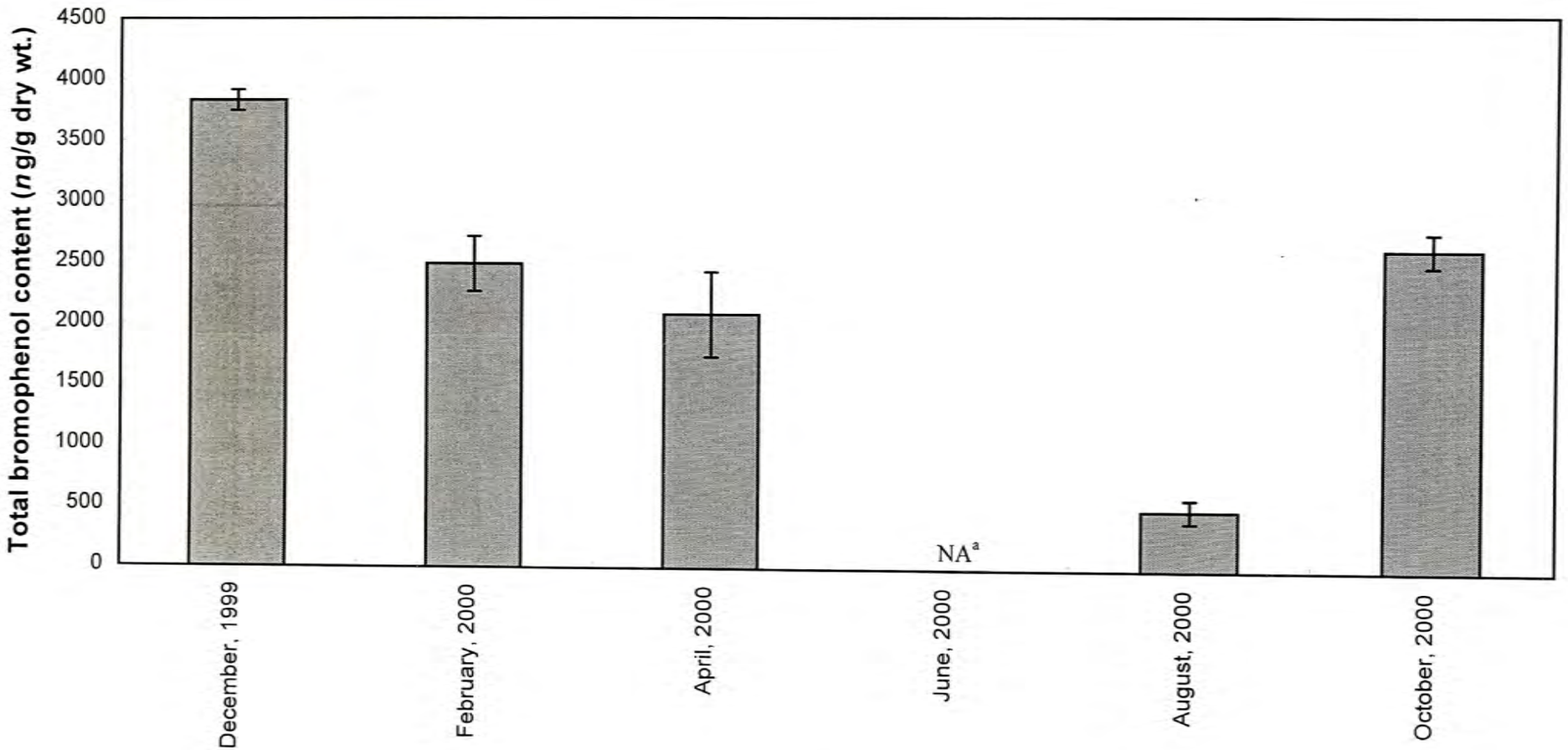
[e]ND = not detected

Figure 4.1. Mean (±SD) total bromophenol content of *Padina arborescens* over time (n=3).



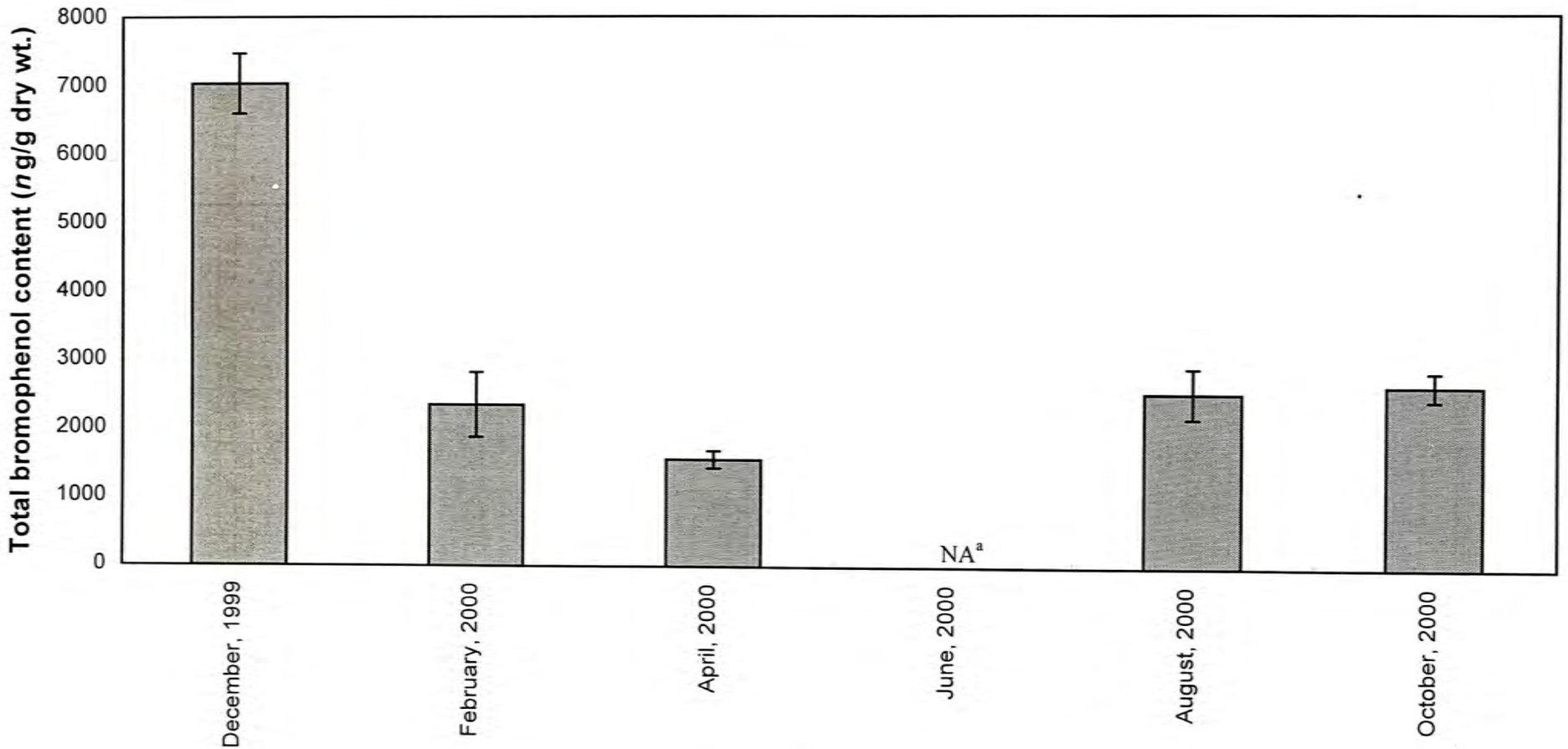
[a]NA=sample not available due to dying back

Figure 4.2. Mean (±SD) total bromophenol content of *Sargassum siliquastrum* over time (n=3).



[a]NA=sample not available due to dying back

Figure 4.3. Mean (±SD) total bromophenol content of *Lobophora variegata* over time (n=3).



[a]NA=sample not available due to dying back

Lobophora variegata, were selected as the samples in this study. The former two algae are members of the marine benthic communities (Sze, 1997) commonly grown on rocks whereas the latter is a brown algae adhered to rocks. They were collected in Tung Ping Chau, an island in the eastern part Hong Kong SAR, China. These species of marine algae were chosen because they grew profusely in the water of Hong Kong. Samples were readily available and could be collected throughout the year of this study, except during the dying back season.

Significant amounts of bromophenols were detected in all samples analyzed. All five bromophenols were found in *Padina arborescens* and *Sargassum siliquastrum*, but only four bromophenols, including 4-BP, 2,4-DBP, 2,6-DBP and 2,4,6-TBP, were found in *Lobophora variegata*. In majorities of the samples investigated, 2,4,6-TBP was present in the highest amount among other bromophenols in the same sample except *Lobophora variegata* in October, 2000 with 2,4-DBP being the highest. In most of the samples, when 2,4,6-TBP was the most abundant one, 2,4-DBP would come next or vice versa. Generally, 2,4-DBP was detected at higher concentrations than 2,6-DBP in all of the samples. These observations were similar to the results obtained by Whitfield *et al.* (1999b) in the survey on marine algae. These results might suggest that bromination at the 2 and 4

positions of the phenolic ring were more favorable than the other positions, and 2,4-DBP was the precursor of 2,4,6-TBP (Whitfield *et al.*, 1999b).

In *Padina arborescens*, the TBCs varied between 40.9 *n*g/g in June, 2000 and 1320 *n*g/g in December, 1999. In August, 2000, there was a seasonal dying back so that samples were not available in that month. Otherwise, all of the five bromophenols were detected throughout the year. Among samples of the same species, 2,4,6-TBP was the most abundant one throughout the year. When comparing the results with that of the other two algae studied, the TBCs in *Padina arborescens* were always the lowest.

The values of TBCs in *Sargassum siliquastrum* fluctuated from 486 *n*g/g (August, 2000) to 3830 *n*g/g (December, 1999). Similarly, sample was unavailable in June, 2000 because of seasonal dying back. Otherwise, the five bromophenols were also detected in all of the *Sargassum siliquastrum* samples. 2,4,6-TBP was present in the highest concentration and 2,4-DBP was the next abundant one. In February and April, 2000, the TBCs in *Sargassum siliquastrum* were the highest among the three species in the same period.

The TBCs detected in *Lobophora variegata* were the highest among the three algae samples except in February and April, 2000. The lowest TBC detected in this species was 1560 *ng*/g in April, 2000 and the highest TBC detected was 7030 ng/g in December, 1999. Dying back of this species occurred in June, 2000. 2,4,6-TBP was the most abundant bromophenol in 4 samples and 2,4-DBP was the highest in October, 2000.

4.3.2 Seasonal variations

The seasonal variations of the TBC in the marine algae studied are shown in Figures 4.1 - 4.3 (p.71-73). All samples had the highest TBCs detected in December, 1999 whereas lower TBCs were generally detected in June and August before the dying back period. Generally, the value of TBCs declined before the algae died back. Dying back occurred in summer (June and August), the season when the temperature was relatively high in Hong Kong. They appeared to decay with loose structure observed before dying back. The biosynthesis of bromophenols might be limited during this time and the bromophenols concurrently diffused from the algae to the surrounding seawater. Thus, the levels of TBCs detected were relatively low. After this period, the algae started to grow again with relatively small structure. Only small amounts of bromophenols were accumulated

in the young body. Thus, the TBCs detected were relatively low. When the algae grew again, the TBCs rose simultaneously.

Similar investigation was carried out on the seasonal variations of bromophenols in *Ulva lactuca* by Flodin *et al.* in Australia (1999). The bromophenol content also showed extreme seasonal variation. Bromophenol concentrations in *Ulva lactuca* were high in late summer (February and March) and low during the rest of the year, an observation that contradicts the results of the present study. In the current study, the bromophenols detected were high in winter (December) and low in summer (June and August). Such conflicting results were due to the differences in locations and climatic conditions where these experiments were performed. This study was performed in Hong Kong located in the northern hemisphere, but the other study was carried out in Sydney, Australia which is located in the southern hemisphere. These two places have totally different seasonal conditions that definitely affect the rate of biosyntheses of various bromophenols. According to the Hong Kong Observatory (2000; 2001) and the Bureau of Meteorology, Commonwealth of Australia (2001, 2002), the average temperature in Australia was relatively lower than that of Hong Kong. In Sydney, the mean temperature in February (summer) and July (winter) was 22.7 °C and 12.7 °C,

respectively. With sub-tropical climate, the average temperature of Hong Kong in August, 2000 (summer) and December, 1999 (winter) was 28.5°C and 16.8°C, respectively. Comparing the two locations, the average temperature of the winter in Hong Kong (December) was similar to that of the summer (February) in Australia. High bromophenol concentrations were detected during winter in Hong Kong but during summer in Australia. It is possible that temperatures around 20°C are suitable for the biosynthesis of the bromophenols in the species of algae studied. Low temperature in the winter (about 13 °C in July) of Australia (Bureau of Meteorology, Commonwealth of Australia, 2002) decreases the production of bromophenols by lowering of the activities of bromoperoxidases in this season (Flodin *et al.*, 1999c). The extremely hot weather in the summer of Hong Kong (about 29 °C in August) is unsuitable for such activities nor for the growth of the marine algae studied. Therefore, dying back occurs every summer in Hong Kong due to overheating and dehydration under strong sunlight (Hodgkiss and Lee, 1983). Thus, biosynthesis of bromophenols is also affected by the temperature. Further investigations should be carried on the effect of temperature on the biosynthesis of this group of compounds.

Interestingly, the seasonal variations of the bromophenol contents of seafood

and marine algae in Hong Kong showed similar patterns [Figures 3.1 – 3.6 (p.42-47), 4.1 – 4.3 (p.71-73)]. The levels of bromophenols were generally lower in summer (June to August) and higher in the rest of the year (October to April) and. However, further investigation should be carried out to find out their relationship.

4.3.3 Functions of bromophenols in marine algae

Marine algae are capable of biosynthesizing bromophenols with the presence of bromoperoxidases (Flodin and Whitfield, 1999a; 1999b) [section 2.2.5.6 (p.21)]. These enzymes catalyze the bromination of phenol and 2-hydroxybenzyl alcohol to 2,4,6-TBP in red algae (Yamada *et al.*, 1985). In green algae, mixtures of the five bromophenols were produced by the bromination of 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol (Flodin and Whitfield, 1999a; 1999b).

Previously, it was believed that these compounds were produced to defense against bacteria, fungi, grazers and competitions (Moore, 1977; Gibson *et al.*, 1979; Reichelt and Borowitzka, 1984; Hornsey and Hide, 1985; Mtolera *et al.*, 1996). However, the results of recent researches and the presence of bromophenols in the grazers showed that these compounds were not an effective anti-predatory nor an anti-bacterial agents (Giray and King, 1997; Lovell *et al.*, 1999). It is still

controversial to conclude on the physiological functions of these compounds in marine algae. When these compounds are consumed by grazers, they are accumulated and inadvertently become a group of the key flavor compounds in the corresponding organisms.

4.3.4 Marine algae as sources of bromophenols in marine environment

In the first part of this project, several seafoods were selected to monitor the distributions of the five targeted bromophenols throughout a year from 1999 to 2000 (chapter 3). These compounds were widely distributed in the Hong Kong seafoods providing desirable ocean-, brine- or iodine-like flavor (Boyle *et al.*, 1992a; Whitfield *et al.*, 1999). The TBCs in guts of the rabbit fish and brown-spotted grouper were higher than those in their flesh which supported the view that the detected bromophenols in the seafoods were likely derived from their diets (Whitfield *et al.*, 1988; 1996; 1997b; 1998). Marine algae, which can synthesize bromophenols, are major dietary choices of some omnivorous (Whitfield *et al.*, 1998). In this survey, we found that the bromophenol concentrations in the marine algae samples were relatively high (TBCs: 40.9 - 7030 *n*g/g). Being food sources of some marine organisms (Lau and Li, 2000), the presence of bromophenols in marine algae in Hong Kong might indicate that these compounds are likely the

sources of these compounds in marine environment.

Marine algae containing abundant amount of bromophenols could be utilized to improve the ocean- or brine-like flavor in the aquacultured products as they usually contain lower amount of these compounds than the wild-harvested ones (Whitfield *et al.*, 1997b; 1999b). Aquacultured prawns contain low amount of bromophenols resulting absence of sea-like flavor due to low levels of bromophenols in their diets (Whitfield et al., 1997b). Seaweed collected in season containing high levels of bromophenols (especially December in Hong Kong with TBCs ranged from 1320 to 7030 *n*g/g dry weight) can be incorporated into the feeds to increase the bromophenol concentrations in the aquacultured products (Whitfield et al., 1997b; Whitfield et al., 1999b) and hence, to improve their flavor quality.

4.4 Conclusion

Bromophenols were present in a relatively high amount in marine algae found in Hong Kong. The TBCs varied between 40.9 and 7030 ng/g on a dry weight basis in the samples. *Lobophora variegata* generally contained higher amounts of bromophenols than other seaweed samples tested. Besides, the bromophenol content showed large seasonal variations, high in winter (about 16°C) and low in

summer (about 28°C) in Hong Kong. The results support that marine algae were one of the sources of bromophenols in marine environment. Further study will evaluate the utilization of seaweed as a source of bromophenols in aquaculture feed.

Chapter 5

Enhancement of bromophenol contents in aquacultured fish by the development of bromophenol-rich fish feeds

5.1 Introduction

The demand for seafood including fish, crustaceans and mollusks from fresh- and seawaters increases every year. The annual worldwide harvest of the fishery products exceeded the 90 million metric limits that the nature can sustain in 1992 (Chen, 2001). It is predicted that there will be a shortage of one hundred billion pounds of fishery products by 2020 due to the decline in the fish population. In order to relief this pressure, many countries utilize aquaculture to increase the productivities and meet the demand (Chen, 2001). Indeed, seafood is one of the most demanded foods consumed in Hong Kong. To increase the fishery production, the government has put much effort into helping the aquaculture sector by providing technical services and credit support (Wei, 1998-1999). In 2000, production from the aquaculture sector was 1770 tonnes valued at HK$102 million (Information Services Department, 2000). The organoliptic quality of these aquacultural products is generally acceptable. But some of the consumers insist that there is obvious difference in flavor between aquacultured and wild marine fishes (Sylvia

and Graham, 1991; Kummer, 1992). Recently, bromophenols were found as a group of flavor compound responsible for such difference in the flavor (Whitfield *et al.*, 1997b). In a study on Australian prawns (Whitfield *et al.*, 1997b), it was found that the concentrations of bromophenols detected in wild-harvested prawns were much higher than that of the cultivated ones. Sensory evaluations showed that the meat of the wild-harvested ones possessed briny, ocean-like and prawn-like flavors whereas the cultivated ones were described as bland. It is believed that the subtle differences between the wild-harvested and the cultivated seafood were caused by the quantitative difference in their bromophenols.

As bromophenols were derived from the diets of the seafood (Whitfield *et al.*, 1997b), it was suggested that the differences of the bromophenol contents in wild-harvested and cultivated prawns were related to the difference in the bromophenol contents of their diets. In the wild-harvested prawns, the dietary components consisted of some bromophenols producing species such as polychaetes with high bromophenol contents. In the cultured seafood, commercial feeds consist of fishmeal, starch, vitamins and minerals supplements. The bromophenols present in these diets were very low (1.4 – 40 ng/g) (Whitfield *et al.*, 1997a; b). Thus, the bromophenol contents of the diets of the seafood seem directly affect their

bromophenol contents.

Previous studies showed that bromophenols were detected in different species of seafoods commonly consumed in Hong Kong (chapter 3), and were presented in high concentrations in several marine algae collected in Hong Kong water (chapter 4). It was suggested that marine algae would be possible dietary sources of bromophenols in some marine animals (Whitfield *et al.*, 1999).

To enhance the quality of the aquacultured fish, their flavor should be improved. A feed that is rich in bromophenol may enhance the bromophenol contents in fish. It would be possible to introduce marine algae into the fish feeds to act as bromophenol sources. In this section, the objectives were (1) to compare the bromophenol content of wild-harvested and cultivated fishes; (2) to develop a fish feed which can increase the bromophenol content in aquacultured fish; and (3) to evualuate the effect of such feeds on the flavor of fish.

5.2 Materials and methods

5.2.1 Preparation of fish feeds

Traditional fish feed and three types of modified fish feeds containing seaweeds

were prepared. The fish feed powder was formulated according to Woo and Kelly (1995) [Table 5.1(p.87)]. To produce the traditional fish feed, fish feed powder was prepared by mixing the ingredients in a plastic container. Afterwards, water was added until a soft dough was formed. The dough was extruded with a Kenwood Large Mincer (A940, PK001/W, Kenwood Limited, U.K.). After extrusion, the feeds were packed and stored at –80°C. The feeds were dried in a freeze dryer for two days to remove excess moisture. Then the fish feeds produced were stored at 4°C.

There were three modified fish feeds tested, which contain 10% and 30% *Padina arborescens*, and 30% *Sargassum siliquastrum* (w/w). To produce the test fish feeds, seaweeds were collected in Tung Ping Chau, Hong Kong SAR, China. Seaweed samples were collected in April, 2000 for the preparation 10% Padina feed, and in October, 2000 for the other two fish feeds. Freshly collected seaweed samples were packed in plastic bags at the collection site, transported to the laboratory immediately and stored in a cold room (4°C). Within 24 hours, they were gently rinsed with tap water. Their rhizoid, sands and living organisms were removed. They were repacked and stored at –80°C. Frozen seaweeds were transferred to a freeze dryer. After four days of drying, the seaweeds were ground

Table 5.1. Composition of fish feed powder (From Woo and Kelly, 1995).

Ingredients	Percentage in Fish Feed (w/w)
White fishmeal	82.66
Dextrin	1.04
Vegetable oil	6
Lard	2.5
Vitamin mix	2
Mineral mix	3.8
Chromic oxide	0.5
Binder[a]	1.5
Total	100

[a]Carboxymethyl cellulose

into powder with a National Blender (MX-T2GM, Matsushita Electric Co. Ltd., Taipei, Taiwan). Both seaweed and normal fish feed powder were mixed in appropriate weight ratios to prepare a dough. Similar preparation procedures as for the above mentioned traditional fish feed were used.

5.2.2 Storage conditions of fish feeds

A preliminary test was performed to evaluate the differences of fish feed stored at different conditions. Fish feed containing 5% *Padina arborescens* was prepared with the method described in section 5.2.1. The feed prepared was packed and stored at three different conditions which were (1) refrigerator (5°C); (2) room condition (20 - 22 °C); and (3) incubator (40 °C). The fish feeds were evaluated after three months by investigating the concentraions of the bromophenols and the moisture of the fish feeds.

5.2.3 Experimental animals

Live aquacultured rabbit fish (*Siganus canaliculatus*) was obtained from local aquaculture farm for the investigations of bromophenol contents. The fish were divided into two portions. Both the gut and the flesh of each fish were collected whereas the head, tail and backbone were discarded.

Silver seabream (*Sparus sarba*) was obtained from local sea cage in Sai Kung. The initial weight of the fish was about 80 g. They were acclimatized and grown in a closed seawater circulating system in the Marine Science Laboratory, the Chinese University of Hong Kong. They received natural photoperiod and were fed with traditional fish feed before the experiment started. Fishes were divided into control and experimental groups, which were fed with traditional feed and feeds containing seaweeds, respectively. About thirty fish were grown for each group. When the fish adapted to the experimental environment (1 – 2 months) with constant eating pattern (once or twice a day) and low lethal rate (<1 fish died each week for each group), fish feeds containing seaweeds were fed to the experimental groups *ad libitum* daily. Three fishes were picked every two weeks to evaluate their bromophenol contents. Two experiments were performed. In the first experiment, traditional fish feed and modified fish feed containing 10% *Padina arborescens* were used to feed the control and experimental group, respectively. In the second experiment, traditional fish feed and two types of modified fish feeds containing 30% *Padina arborescens*, and 30% *Sargassum siliquastrum* were used to feed the control and two experimental groups, respectively.

5.2.4 Solvents and chemicals

Organic solvent and standard samples of the five target bromophenols were purchased from Lab-scan Ltd. (Ireland), Aldrich Chemical Co. (Milwaukee, WI) and Acros Organics (Belgium). The purities of the five bromophenols ranged from 97% to 99%.

5.2.5 Extraction and quantification of bromophenols

5.2.5.1 Simultaneous steam distillation-solvent extraction (SDE)

The extraction method was adapted from Whitfield *et al.* (1988, 1997b). Each sample was homogenized by a National Blender (MX-T2GM, Matsushita Electric Co. Ltd., Taipei, Taiwan) for four minutes. Treated samples, 1 mL of internal standard (1,3,5-trimethylbenzene, concentration at 84.7 μg/mL) and 500 mL of boiled double distilled water were transferred to a 5-L round bottom flask. The sample was then acidified to pH 1 with 96% sulphuric acid. Extraction with 40-mL of pentane/diethyl ether (9:1 v/v) for 2.5 hours in a Likens and Nickerson type SDE apparatus (Cat. No. K-523010-0000, Kontes, Vineland, NJ) was then carried out. The pH of the residue after extraction was measured again to ensure that the acidity was maintained throughout the process. Triplicate extractions of each sample were carried out. Extract collected was further concentrated to 0.25 mL with a stream of

ultra high purity (99.999%) nitrogen and dried over 2.85 g anhydrous sodium sulfate. The concentrated extract was temporary stored in a 15-mL conical tube at –80°C until further analyses were carried out.

5.2.5.2 Gas chromatography/mass spectrometry (GC/MS)

A GC/MS system consisting of a Hewlett-Packard 6890 GC coupled with a HP 5973 mass selective detector (MSD) (Hewlett-Packard Co., Palo Alto, CA) was used for qualitative and quantitative analyses. Five μL of each extract was injected, in splitless mode with injector temperature at 200°C, into a fused silica open tubular column (Supelcowax-10, 60 m length × 0.25 mm i.d. × 0.25 μm film thickness; Supelco, Inc., Bellefonte, PA). Helium gas (ultra high purity grade, 99.999%) was used as carrier gas at constant linear velocity at 30 cm/s. Oven temperature was programmed from 100 to 200°C at a ramp rate of 10°C/min. The initial and final hold times were 5 and 75 mins., respectively. MS interface, ion source and MS quadrupole temperatures were set at 250°C, 230°C and 106°C, respectively. Ionization voltage was 70 eV and electron multiplier voltage was 1200 V. Selected ion monitoring (SIM) GC/MS procedure was used. Ions were monitored for 2- and 4-bromophenol (2- and 4-BP) at *m/z* 172 and 174; for 2,4- and 2,6-dibromophenol (2,4- and 2,6-DBP) at *m/z* 250 and 252; for 2,4,6-tribromophenol

(2,4,6-TBP) at *m/z* 330 and 332; and for internal standard 1,3,5-trimethylbenzene at *m/z* 105 and 120 (Lee *et al.*, 1984; Whitfield *et al.*, 1997b; Chung, 1999).

5.2.5.3 Compound identification and quantification

The presence of each bromophenol was confirmed by the detection of a single peak in the selected ion chromatogram at corresponding retention time and by the presence of the two characteristic ions listed above with particular isotopes ratios (Whitfield *et al.*, 1997b).

For quantification, 3-point standard curves for each bromophenol were established. Solutions (5mL) containing 5 mg of each of the five bromophenols was prepared. Serial dilutions at ratios of 1:5 and 1:25 were made. A constant amount of internal standard, 1,3,5-trimethylbenzene (5 mg), was added into each of the above solutions prepared. The area ratios from both selected ions (bromophenol/internal standard) were plotted against the amount ratios to obtain a response factor for each bromophenol. Ions chosen were with *m/z* of 172 (monobromophenols), 252 (dibromophenols), 330 (tribromophenol) and 105 (internal standard). When response factor of each compound was obtained, the amount ratios (bromophenol/internal standard) of each bromophenol in the extracts

could be calculated. Concentration of a bromophenol in a sample was calculated by the following equation (Equation 1):

Concentration of a bromophenol (*n*g/g dry weight)

$$= \frac{\text{(amount ratio of bromophenol/internal standard)} \times \text{amount of internal standard } (n\text{g})}{\text{dry weight of sample (g)}}$$

.....Equation 1

The total bromophenol content (TBC) was calculated by the following equation (Equation 2):

Total bromophenol content (TBC) (*n*g/g dry weight)

=Summation of the concentrations of 2-BP, 4-BP, 2,4-DBP, 2,6-DBP and 2,4,6-TBP (*n*g/g)

.....Equation 2

5.2.5.4 Recoveries

The recoveries of the SDE technique on the various bromophenols were calculated according to Equation 3. Briefly, it was determined by extracting known amount of each bromophenol with the SDE method, and quantified with the same GC/MS system under the same experimental conditions described above. These values were used to calculate the original amount of bromophenols in the samples. The average recoveries of the bromophenols were listed in chapter 3 (p.35).

Recovery (%)

$$= \frac{\text{Concentration of bromophenol detected}}{\text{Concentration of bromophenol standard added}} \times 100\% \quad \text{.....Equation 3}$$

5.2.6 Moisture determination

The percentage moisture of each of the samples was determined using a Mettler LJ16 moisture analyzer (Mettler-Toledo, Switzerland). Dry weights of the samples were then determined. Concentrations of the bromophenols in the samples were expressed on a dry weight basis.

5.2.7 Statistical analysis

TBCs were compared between the gut and the flesh of the fish, and between the flesh of wild-harvested and the aquacultured fishes by *t*-tests at p=0.05 level of significance. TBCs in different fish feeds were analyzed by one-way analysis of variance (ANOVA) and compared by the Tukey test at p=0.05 level of significance. Besides, the TBCs in the fish meats and the weight of fish fed with different fish feeds were analyzed by two-way ANOVA at level of significance of p=0.05.

5.2.8 Sensory tests

The sensory differences between the fishes in the control group (fed with traditional fish feed) and in two experimental groups (fed with 30% *Padina arborescens* and 30% *Sargassum siliquastrum* modified fish feeds, respectively) were evaluated by Triangle tests. Twenty-two subjects were invited to participate in the sensory evaluation (Meilgaard *et al.*, 1991). Orientation and training were provided before testing the fish samples to make the subjects familiar with the flavor of bromophenols and the procedures involved in the Triangle test. During training, subjects received different test solutions containing different concentrations of bromophenols near their threshold values in water. They were asked to differentiate among solutions containing different concentrations.

During the actual sample test, subjects were physically isolated from each other to minimize disturbances in a clean and cooled room (20°C). Also, red light was used by covering a red filter in front of the light source (Meilgaard *et al.*, 1991). Samples were prepared in advance and served immediately after the subjects arrived.

Each subject performed four tests, two for comparing the fish flesh from the control group and the group fed with feed containing 30% *Padina arborescens*, and

two for comparing the fish flesh from the control group and the group fed with feed containing 30% *Sargassum siliquastrum*. In each test, there were three cubes of sample fish meat (1cm × 1cm × 1cm) wrapped with aluminum foil which had been steamed for 15 minutes. Samples were kept in paper cups labeled with random numbers. Samples were sniffed by nose and tasted by mouth. Subjects were not allowed to swallow the samples but required to rinse their mouth between each evaluation. Subjects were asked to pick out one sample which was different from the other two samples. They were required to write down their comments on the samples. Published table (Roessler *et al.*, 1978) was used to determine the minimum correct number of identifications required to obtain a statistically significant difference ($p<0.05$) and to compare with the result to draw a conclusion (Gillette, 1994).

5.3 Results and discussion

5.3.1 Bromophenol contents in wild-harvested and aquacultured fish

The distributions of the bromophenols of aquacultured rabbit fish (*Siganus canaliculatus*) are shown in Table 5.2 (p.97). 2-BP, 2,4-DBP and 2,4,6-TBP were found in the gut of the fish samples. 2,4,6-TBP was present at the highest concentration among the three compounds (4.92 *n*g/g) in the gut and was the only

Table 5.2. Distribution of bromophenols in wild-harvested and aquacultured rabbit fish (*Siganus canaliculatus*).

	Bromophenol Concentration (*n*g/g dry wt.)					
	2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]	TBC[b]
Siganus canaliculatus						
Wild-harvested						
Gut	7.82[c] ± 0.35[d]	ND [e]	7.62 ± 1.55	7.42 ± 1.67	26.9 ± 2.0	49.7 ± 5.2
Flesh	10.7 ± 2.9	ND	0.727 ± 0.192	0.555 ± 0.050	27.4 ± 3.3	39.4 ± 4.7
Aquacultured						
Gut	0.659 ± 0.019	ND	1.68 ± 0.25	ND	4.92 ± 1.81	7.26 ± 1.86
Flesh	ND	ND	ND	ND	1.70 ± 0.10	1.70 ± 0.10

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol

[b]Total bromophenol content

[c]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates

[d]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)

[e]ND = not detected

bromophenol detected in the flesh (1.70 *n*g/g).

The bromophenol contents of the aquacultured rabbit fish were compared with the wild-harvested ones collected in the same month (December, 2000) to determine if the sources of fish had any effect on the bromophenol contents. Comparing the two samples, the TBCs in the wild-harvested sample were significantly higher in both the gut and the flesh than those in the aquacultured one compared by t-test ($p<0.05$). The TBC in the gut of the wild-harvested one was 49.7 *n*g/g and that in the flesh was 39.4 *n*g/g, while that in the gut and flesh of the aquacultured one was 7.26 and 1.70 *n*g/g, respectively.

Such observations were consistent with that of Whitfield *et al.* (1997b) in that the bromophenols contents in the wild-harvested animal, e.g. prawns, were higher than those in the cultured ones. They suggested that the differences were caused by the difference in the bromophenol contents in their diet. Natural prawn diet contained polychaetes with high amount of bromophenols (Whitfield *et al.*, 1999b) while commercial prawn feeds contain very low amount of bromophenols. The aquacultured rabbit fish analyzed in this experiment was fed with commercial feed. Owing to this, the bromophenol contents in the aquacultured rabbit fish were

relatively lower.

The wild-harvested prawns were described as having "prawn-like" and "ocean-like" flavor while the cultivated ones were reported to be sweet but bland (Whitfield *et al.*, 1997b). It is believed that the lack of sea-like flavor in various aquacultured seafoods (Sylvia and Graham, 1991; Kummer, 1992) was due to the low concentrations of bromophenols they contained. In order to increase the acceptability and the value of the aquacultured products, the bromophenol contents within them should be increased to improve their flavor quality. In the next section, methods were described and tested in aquacultured fishes to increase their bromophenol contents by modifying the compositions of the feeds.

5.3.2 Development of bromophenol-rich fish feed

There was much evidence supporting that bromophenols were obtained from diets by the marine animals. The relatively higher levels of the bromophenol contents in the gut of fish and the head of prawns collected in Australia (Whitfield *et al.*, 1997b; 1998) strongly support this hypothesis. Similar results were observed in the fish and the prawns found in Hong Kong (chapter 3). Besides, the loss of the sea-like flavor of Pacific salmon during transition from seawater to freshwater

habitats suggested that the loss of bromophenols was due to the lack of diet containing sufficiently high bromophenol in the freshwater environment (Boyle *et al.*, 1992a). Whitfield *et al.* (1988) suggested that the extraction of the bromophenols was more favorable in acidic environment such as in the fish stomach. Bromophenols would likely be released in the stomach, and subsequently absorbed through the gut. To improve the flavor quality of the aquacultured fish products, concentrations of the bromophenols in the diets should be increased. Bromophenols absorbed might accumulate in the flesh to intensify the desirable sea-like or ocean-like flavor. The most direct method to increase them was to add those compounds to the fish diet but it failed because bromophenols were lost during processing of the feed (Whitfield *et al.*, 1997a). Alternately, it might be possible to produce feeds containing natural bromophenol sources.

The surveys showed that the Australian marine algae and polychaetes (Whitfield *et al.*, 1999a; b) contained high levels of bromophenols because they are able to produce bromophenols by the action of bromoperoxidases. In the previous chapter, three types of marine algae commonly found in Hong Kong were analyzed on their bromophenol contents. Results showed that their bromophenol concentrations were high. Thus, they could be a suitable source to produce fish

feeds containing bromophenols.

In the first experiment, both traditional and modified fish feeds were produced, and the retentions of bromophenols within them were evaluated. Traditional fish feed was produced to feed the control group of fishes. For the modified fish feed, marine alga *Padina arborescens* was chosen which had a TBC of 1130 *n*g/g to feed the experimental fishes. The alga was selected due to its availability and abundance most of the time throughout a year. *Padina* species were potential dietary sources of protein and lipid for fish (Wahbeh, 1997). Ten percent (w/w) of the dry *Padina arborescens* powder was mixed with the powder of fish feed. Distributions of the bromophenols in the two feeds are shown in Table 5.3 (p.102).

The TBC in the traditional fish feed was 7.77 *n*g/g and that of the modified one was 39.4 *n*g/g. The TBC in the modified fish feed with 10% *Padina arborescens* was significantly higher than that of the traditional fish feed compared by t-test ($p<0.05$). The difference was 5.07 fold. All of the five bromophenols were detected in the modified fish feed with 2,4-DBP present in the highest concentration. In the traditional fish feed, 2- and 4-BP were not detected.

Table 5.3. Distribution of bromophenols in fish feeds used in the 1st experiment.

Bromophenol Concentration (*n*g/g dry wt.)						
2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]	TBC[b]	Sig[f]
Traditional Fish Feed						
ND [e]	ND	2.60[c] ± 0.82[d]	1.85 ± 0.49	3.32 ± 1.08	7.77 ± 2.35	
Modified Fish Feed (10% *Padina arborescens*)						*
1.32 ± 0.32	6.39 ± 0.72	14.3 ± 3.2	7.60 ± 3.85	9.80 ± 1.30	39.4 ± 9.3	

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol

[b]Total bromophenol content

[c]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates

[d]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)

[e]ND = not detected

[f]* statistically significant different ($p<0.05$) in TBC between fish feeds compared by t-test

In order to study the effects of varying both the amount and the species of the marine algae in the feeds on the bromophenol contents, and on the bromophenol contents of the fishes fed with them, a second experiment was carried out. Fishes fed with the traditional fish feed was the control group. Two kinds of modified feeds were produced by mixing 30% (w/w) of the dry *Padina arborescens*, and 30% (w/w) of the dry *Sargassum siliquastrum* with the fish feed powder. The distributions of the bromophenols in the feeds are shown in Table 5.4 (p.104).

The TBC of the traditional fish feed was 8.90 *n*g/g in this experiment, and the TBCs in the modified feeds containing 30% *Padina arborescens* and 30% *Sargassum siliquastrum* were 132 and 340 *n*g/g, respectively. Statistical test (one-way ANOVA) showed that the three types of feeds were significantly different from each other ($p<0.05$). The bromophenol contents in the modified feed with 30% *Sargassum siliquastrum* was higher than that of the 30% *Padina arborescens*. All of the five bromophenols were detected in the two modified feeds.

Preliminary shelflife evaluations of the fish feeds produced had been performed in order to select a suitable storage condition for the feeds before the experiment started. The feeds containing 5% *Padina arborescens* were stored in different

Table 5.4. Distribution of bromophenols in fish feeds used in the 2nd experiment.

Bromophenol Concentration (*n*g/g dry wt.)						
2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]	TBC[b]	Sig[f]
Traditional Fish Feed						
ND [e]	ND	3.01[c] ± 1.58[d]	2.02 ± 1.03	3.87 ± 0.91	8.90 ± 3.10	A
Modified Fish Feed (30% *Padina arborescens*)						
10.8 ± 3.7	37.0 ± 17.7	44.5 ± 24.9	12.1 ± 2.5	27.8 ± 13.5	132 ± 62	B
Modified Fish Feed (30% *Sargassum siliquastrum*)						
3.88 ± 1.23	9.17 ± 1.82	67.6 ± 13.9	7.39 ± 0.59	252 ± 37	340 ± 42	C

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol

[b]Total bromophenol content

[c]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates

[d]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)

[e]ND = not detected

[f]Values of average TBCs in different types of fish feeds marked with superscripts (A-C) are significantly different (Tukey, $p<0.05$)

temperature and the qualities were analyzed after three months. Values of the TBCs of the feeds stored at different conditions were compared by one-way ANOVA test. The result [Table 5.5 (p.106)] showed that the best storing condition was packed and stored in 4°C refrigerator. There was no significant difference between the TBC values of the feed at the beginning and that stored at 4 °C after three months ($p>0.05$). Bromophenols and the moisture of the feeds could be retained under this condition. For fish feeds stored at room temperature and incubator, the TBCs decreased significantly ($p<0.05$) and bromophenols within the fish feeds were lost. Thus, all of the fish feeds were packed and stored at 4°C before being used to feed the fish.

5.3.3 Effect of feeding the fish with the fish feed developed

In this experiment, silver seabream (*Sparus sarba*) was chosen as the experimental fish [section 2.3(p.24)]. The main reasons included the availability of the juvenile fish from local sea cage. Besides, techniques and formulation of the feed for seabream are well developed (Woo and Kelly, 1995). Therefore, the possibility of successfully carrying out this experiment was high.

In the first experiment, the fish were divided into two groups in two tanks. One of them was the control group fed with the traditional feed whereas the other

Table 5.5. Total bromophenol content and moisture content of fish feed stored at different conditions.

Time (month)	Storage Condition	Storage Temperature (°C)	TBC [a] (*n*g/g dry wt.)	Sig[d]	Moisture Content (%)
0 (Control)	-	-	49.7[b] ± 1.1[c]	A	4.3
3	Refrigerator	5	49.6 ± 1.3	A	5.0
3	Room Temperature	20 - 22	38.8 ± 6.1	B	5.0
3	Incubator	40	24.7 ± 1.9	C	3.9

[a]TBC=Total bromophenol content

[b]Average TBC (*n*g/g dry wt.) from 3 replicates

[c]Standard deviation of the TBC (*n*g/g dry wt.)

[d]Values of average TBCs in different types of fish feeds marked with superscripts (A-C) are significantly different (Tukey, $p<0.05$)

one was the experimental group fed with the modified feed containing 10% *Padina arborescens*. Fish samples were collected every two weeks and their bromophenol contents in the gut and the flesh were evaluated. Variations of the concentrations of the bromophenols are shown in Tables 5.6 and 5.7 (p.108 and 109), and Figure 5.1 (p.110). 2,4-DBP and 2,4,6-TBP were detected in all of the samples and were present with the highest concentration in most of the samples. TBCs in gut were higher than that in the flesh in all of the samples. Besides, there was a slight decrease in the TBC at the beginning four weeks. This might due to some environmental factors such as change in water temperature or disturbance during experiment such as sampling that affected the eating pattern of the fish.

From the results of two-way ANOVA tested on the average TBC values of the two groups of fish fed with traditional feed and modified feed [Table 5.8 (p.111)], a significant difference was found ($p<0.05$). However, the difference in changes of the average TBCs among the two groups with time was not statistically significant ($p>0.05$). Feed containing 10% Padina arborescens could not significantly increase the bromophenol contents in the flesh of fish fed with it after eight weeks. Nevertheless, the average TBCs of the experimental group was relatively higher, the feed with 10% *Padina arborescens* powder might have some effect on the

Table 5.6. Distribution of bromophenols in the gut and the flesh of silver seabream fed with traditional fish feed for 8 weeks (1st experiment).

Sample		Bromophenol Concentration (*n*g/g dry wt.)						
		2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]	TBC[b]	Sig[c]
Feed used:	**Traditional fish feed**							
Week 0	gut	ND [d]	ND	35.4[e] ± 8.9[f]	ND	34.1 ± 7.9	69.5 ± 16.4	*
	flesh	ND	ND	3.85 ± 1.81	ND	5.85 ± 3.21	9.70 ± 4.85	
Week 2	gut	ND	ND	5.91 ± 1.42	ND	24.7 ± 13.1	30.6 ± 14.5	*
	flesh	ND	ND	0.921 ± 0.710	ND	ND	0.921 ± 0.710	
Week 4	gut	ND	ND	13.6 ± 6.4	3.01 ± 0.04	25.8 ± 5.5	42.4 ± 11.0	*
	flesh	ND	ND	2.39 ± 0.70	ND	ND	2.39 ± 0.70	
Week 6	gut	ND	ND	17.8 ± 8.1	ND	44.6 ± 35.3	62.3 ± 43.3	*
	flesh	ND	ND	2.84 ± 1.04	ND	2.76 ± 1.65	5.60 ± 2.63	
Week 8	gut	ND	ND	8.48 ± 1.40	4.96 ± 1.24	110 ± 54	123 ± 57	*
	flesh	ND	ND	6.34 ± 6.85	ND	6.02 ± 1.46	12.4 ± 7.51	

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol

[b]Total bromophenol content (*n*g/g dry wt.)

[c]*Statistically significant difference ($p < 0.05$) between TBC in gut and in flesh compared by t-test

[d]ND=not detected

[e]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates

[f]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)

Table 5.7. Distribution of bromophenols in the gut and the flesh of silver seabream fed with fish feed containing 10% *Padina arborescens* for 8 weeks (1st experiment).

Sample		Bromophenol Concentration (*n*g/g dry wt.) 2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]	TBC[b]	Sig[c]
Feed used:	**Modified Fish Feed (10% *Padina arborescens*)**							
Week 0	gut	ND [d]	ND	35.4[e] ± 8.9[f]	ND	34.1 ± 7.9	69.5 ± 16.4	*
	flesh	ND	ND	3.85 ± 1.81	ND	5.85 ± 3.21	9.70 ± 4.85	
Week 2	gut	ND	ND	14.1 ± 5.9	ND	73.4 ± 68.0	87.5 ± 73.9	-
	flesh	ND	ND	4.22 ± 1.15	ND	ND	4.22 ± 1.15	
Week 4	gut	ND	ND	48.2 ± 3.2	ND	84.0 ± 9.6	132 ± 13	*
	flesh	ND	ND	2.60 ± 1.03	ND	ND	2.60 ± 1.03	
Week 6	gut	ND	ND	339 ± 218	ND	252 ± 124	591 ± 342	*
	flesh	ND	ND	7.23 ± 4.05	ND	5.85 ± 2.06	13.1 ± 6.1	
Week 8	gut	27.7 ± 7.2	ND	74.9 ± 17.9	13.1 ± 1.7	492 ± 62	608 ± 84	*
	flesh	0.916 ± 0.335	ND	2.51 ± 0.21	ND	19.9 ± 5.8	23.3 ± 6.2	

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol

[b]Total bromophenol content (*n*g/g dry wt.)

[c]*Statistically significant difference (p<0.05) between TBC in gut and in flesh compared by t-test, - no statistically significant difference (p>0.05)

[d]ND=not detected

[e]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates

[f]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)

Figure 5.1. Mean (±SD) total bromophenol contents in the flesh of silver seabream fed with different fish feeds (1st experiment).

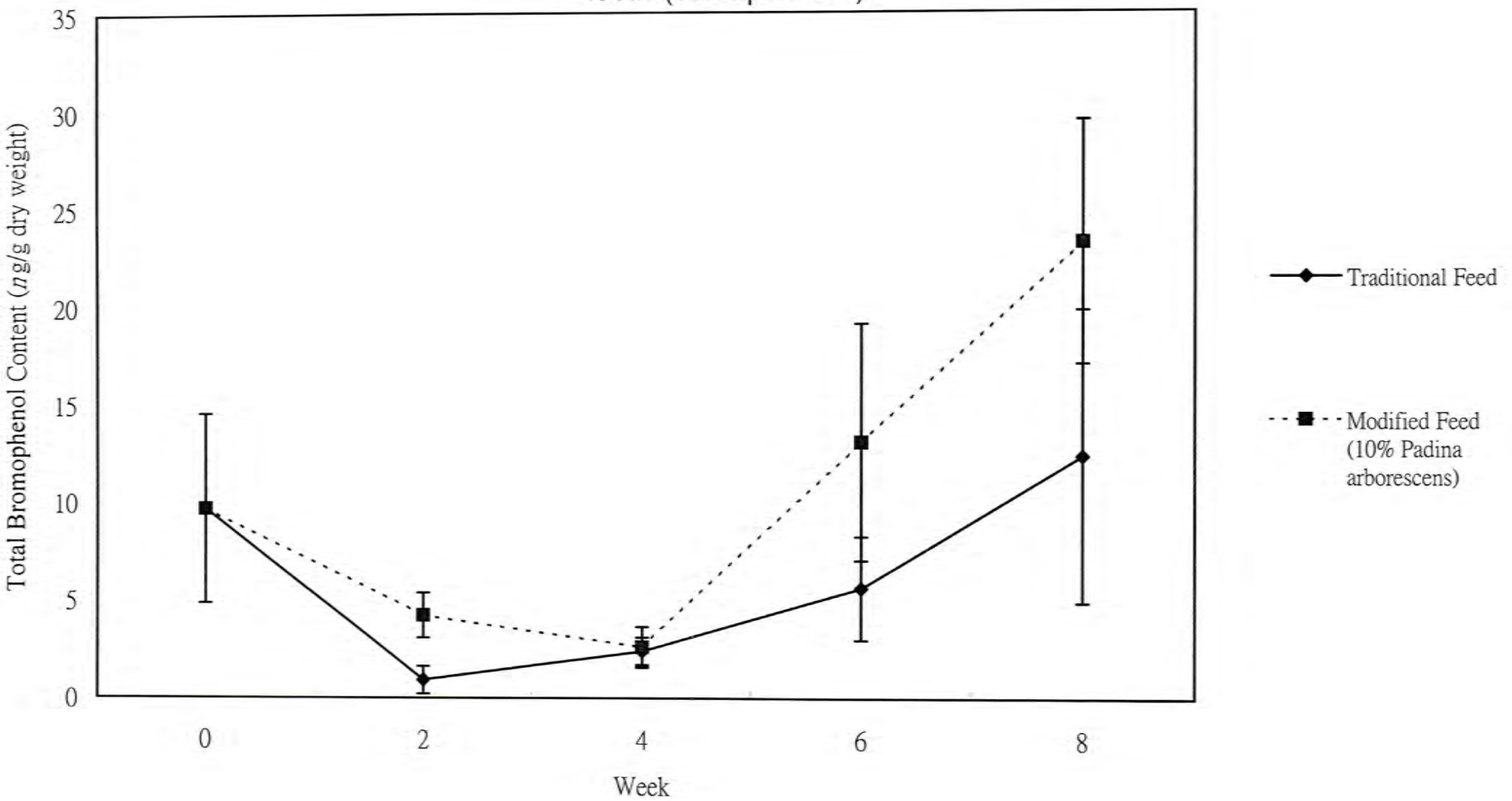


Table 5.8. Results of the two-way ANOVA for the total bromophenol content in the flesh of fish fed with different diets after 8 weeks (1st experiment).

Control Group:	Traditional feed	
Experimental Group:	Feed containing 10% *Padina arborescens*	
Source of Variation	***p*-value**	**Sig[a]**
Between Groups (control and experimental)	0.012	*
Time	<0.001	*
Interaction (Group × Time)	0.168	-

[a] * statistically significant difference ($p<0.05$) in the factor indicated among samples;
- no statistically significant difference ($p>0.05$) among samples

bromophenols contents in the fish. In the second experiment, fish feeds mixed with higher proportion of algae were used to determine if more significant results could be obtained.

In the second experiment, the fish were divided into three groups (about 30 fish per group) grown in three different tanks. One of them were the control group fed with traditional fish feed, and the other two group were the experimental groups fed with modified feeds mixed with 30% *Padina arborescens* and 30% *Sargassum siliquastrum*, respectively. Feed containing 30% *Padina arborescens* was used to evaluate the effect of increasing the amount of seaweed in the feed on the bromophenol contents of the experimental fishes when comparing with that in the first experiment. Feed containing 30% *Sargassum siliquastrum* was used to study the effect of using different species of algae in the feed. Samples were also collected at a two-week interval. The distributions of the bromophenols in the fish samples are shown in Table 5.9 to 5.11 (p.113-115) and Figure 5.2 (p.116). 2,4-DBP and 2,4,6-TBP were detected in all of the samples. 2,4,6-TBP was present in the highest concentrations among the five bromophenols in most of the samples. TBCs in gut were higher than that in flesh.

Table 5.9. Distribution of bromophenols in the gut and the flesh of silver seabream fed with traditional fish feed for 8 weeks (2nd experiment).

Sample		Bromophenol Concentration (*n*g/g dry wt.)						
		2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]	TBC[b]	Sig[c]
Feed used:	**Traditional fish feed**							
Week 0	gut	ND [d]	ND	14.1[e] ± 8.2[f]	ND	88.9 ± 8.0	103 ± 5	*
	flesh	ND	ND	5.60 ± 1.29	ND	33.4 ± 7.4	39.0 ± 8.5	
Week 2	gut	ND	ND	32.6 ± 8.6	ND	188 ± 47	221 ± 55	*
	flesh	ND	ND	3.56 ± 0.46	ND	21.3 ± 6.4	24.9 ± 6.8	
Week 4	gut	ND	ND	10.5 ± 8.9	ND	28.9 ± 17.6	39.3 ± 26.5	-
	flesh	ND	ND	3.61 ± 1.07	ND	20.0 ± 5.2	23.6 ± 6.1	
Week 6	gut	ND	ND	9.23 ± 2.30	ND	38.4 ± 12.5	47.6 ± 13.1	-
	flesh	ND	ND	4.30 ± 2.61	ND	31.0 ± 21.1	35.3 ± 23.7	
Week 8	gut	ND	ND	15.5 ± 6.0	31.1 ± 7.4	110 ± 44	156 ± 51	*
	flesh	ND	ND	13.2 ± 5.4	ND	26.0 ± 4.9	39.2 ± 9.4	

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol

[b]Total bromophenol content (*n*g/g dry wt.)

[c]*Statistically significant difference ($p<0.05$) between TBC in gut and in flesh compared by t-test, - no statistically significant difference ($p>0.05$)

[d]ND=not detected

[e]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates

[f]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)

Table 5.10. Distribution of bromophenols in the gut and the flesh of silver seabream fed with fish feed containing 30% *Padina arborescens* for 8 weeks (2nd experiment).

Sample		Bromophenol Concentration (*n*g/g dry wt.) 2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]	TBC[b]	Sig[c]
Feed used:	**Modified Fish Feed (30% *Padina arborescens*)**							
Week 0	gut	ND [d]	ND	14.1[e] ± 8.2[f]	ND	88.9 ± 8.0	103 ± 5	*
	flesh	ND	ND	5.60 ± 1.29	ND	33.4 ± 7.4	39.0 ± 8.5	
Week 2	gut	23.6 ± 17.7	370 ± 139	92.6 ± 31.1	ND	423 ± 236	909 ± 141	*
	flesh	3.19 ± 2.06	ND	7.77 ± 2.87	ND	36.1 ± 19.8	47.1 ± 24.5	
Week 4	gut	56.6 ± 29.6	ND	286 ± 106	ND	895 ± 443	1240 ± 580	*
	flesh	9.60 ± 8.57	ND	21.4 ± 12.4	ND	75.1 ± 45.7	106 ± 66	
Week 6	gut	37.8 ± 24.9	ND	101 ± 29	ND	289 ± 115	428 ± 162	*
	flesh	6.02 ± 2.78	ND	11.1 ± 6.4	ND	69.7 ± 35.8	86.9 ± 44.8	
Week 8	gut	12.6 ± 4.3	ND	37.9 ± 7.5	ND	123 ± 12	173 ± 14	*
	flesh	8.06 ± 0.66	ND	10.7 ± 3.1	ND	57.5 ± 8.8	76.3 ± 10.0	

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol

[b]Total bromophenol content (*n*g/g dry wt.)

[c]*Statistically significant difference (p <0.05) between TBC in gut and in flesh compared by t-test

[d]ND=not detected

[e]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates

[f]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)

Table 5.11. Distribution of bromophenols in the gut and the flesh of silver seabream fed with fish feed containing 30% *Sargassum siliquastrum* for 8 weeks (2nd experiment).

Sample		Bromophenol Concentration (*n*g/g dry wt.) 2-BP[a]	4-BP[a]	2,4-DBP[a]	2,6-DBP[a]	2,4,6-TBP[a]	TBC[b]	Sig[c]
Feed used:		**Modified Fish Feed (30% *Sargassum siliquastrum*)**						
Week 0	gut	ND [d]	ND	14.1[e] ± 8.2[f]	ND	88.9 ± 8.0	103 ± 5	*
	flesh	ND	ND	5.60 ± 1.29	ND	33.4 ± 7.4	39.0 ± 8.5	
Week 2	gut	11.9 ± 6.3	ND	63.4 ± 19.2	ND	502 ± 267	577 ± 292	*
	flesh	ND	ND	13.8 ± 3.2	ND	68.5 ± 47.6	82.3 ± 49.6	
Week 4	gut	ND	ND	219 ± 39	ND	310 ± 38	529 ± 76	*
	flesh	ND	ND	8.40 ± 3.69	ND	51.7 ± 19.6	60.1 ± 21.1	
Week 6	gut	19.3 ± 4.7	ND	31.3 ± 4.5	32.7 ± 12.5	126 ± 16	209 ± 28.6	*
	flesh	ND	ND	32.3 ± 23.4	ND	74.5 ± 15.4	107 ± 37	
Week 8	gut	25.9 ± 9.9	ND	74.8 ± 49.0	75.4 ± 72.5	330 ± 115	506 ± 241	-
	flesh	11.9 ± 6.2	ND	16.9 ± 2.9	ND	105 ± 28	134 ± 31	

[a]2-BP: 2-bromophenol; 4-BP: 4-bromophenol; 2,4-DBP: 2,4-dibromophenol; 2,6-DBP: 2,6-dibromophenol; 2,4,6-TBP: 2,4,6-tribromophenol

[b]Total bromophenol content (*n*g/g dry wt.)

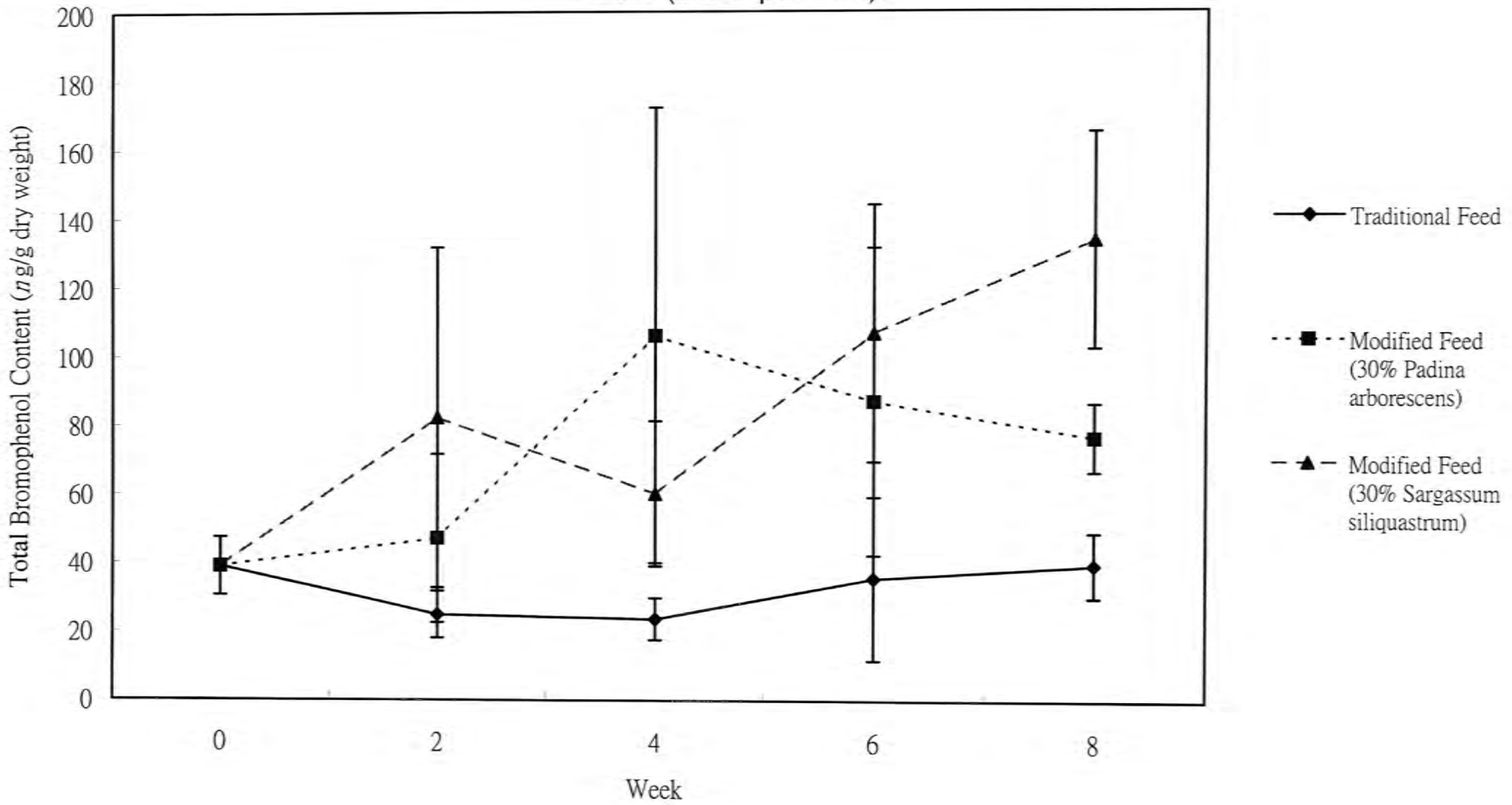
[c]*Statistically significant difference (p<0.05) between TBC in gut and in flesh compared by t-test, - no statistically significant difference (p>0.05)

[d]ND=not detected

[e]Average bromophenol concentration (*n*g/g dry wt.) from 3 replicates

[f]Standard deviation of the bromophenol concentration (*n*g/g dry wt.)

Figure 5.2. Mean (±SD) total bromophenol contents in the flesh of silver seabream fed with different fish feeds (2nd experiment).



For the group of fish fed with modified feed mixed with 30% *Padina arborescens*, the two-way ANOVA result [Table 5.12 (p.118)] showed that there was a significant difference between the average TBCs of the experimental and the control groups ($p<0.05$). The average TBCs in the experimental group was higher than that of the control group. However, the difference of the average TBCs in the two groups with time was not significant ($p>0.05$). Feed containing 30% Padina arborescens could not significantly increase the bromophenol contents in the flesh of fish fed with it after eight weeks. Compared this group with that in the first experiment (feed containing 10% *Padina arborescens*), the final TBC of fish flesh in the group fed with feed mixed with 30% *Padina arborescens* (76.3 *n*g/g) was higher than that of the group fed with feed containing 10% *Padina arborescens* (23.3 *n*g/g). This could be explained by larger amount of bromophenol contained in the feed mixed with 30% *Padina arborescens* (132 *n*g/g) than that with 10% *Padina arborescens* (39.4 *n*g/g).

In the group fed with modified feed mixed with 30% *Sargassum siliquastrum*, the difference between the mean values of TBCs of the experimental and the control group was significant ($p<0.05$) when compared by two-way ANOVA test [Table 5.12 (p.118)]. Moreover, the difference in the mean values among the two groups along

Table 5.12. Results of the two-way ANOVA for the total bromophenol content in the flesh of fish fed with different diets after 8 weeks (2nd experiment).

Control Group:	Traditional feed		Traditional feed	
Experimental Group:	Feed containing 30% *Padina arborescens*		Feed containing 30% *Sargassum siliquastrum*	
Source of Variation	***p*-value**	**Sig[a]**	***p*-value**	**Sig[a]**
Between Groups (control and experimental)	0.001	*	<0.001	*
Time	0.293	-	0.016	*
Interaction (Group × Time)	0.165	-	0.037	*

[a] * statistically significant difference ($p<0.05$) in the factor indicated among samples;
- no statistically significant difference ($p>0.05$) among samples

with time were statistically significant at $p<0.05$. The TBCs in the fish fed with modified feed was greater than those fed with traditional feed. The TBC in the experiment group increased with time that caused significant difference with the control group. The modified feed mixed with 30% *Sargassum siliquastrum* could successfully increase the bromophenol contents in the aquacultured fish through accumulation after eight weeks. TBC detected in the flesh of fish in this group was increased to 134 *n*g/g.

The TBC in the fish feed containing 30% *Sargassum siliquastrum* was 340 *n*g/g whereas that of the fish feed containing 30% Padina arborescens was 132 *n*g/g. The TBC in the former was 2.58 times higher than the later one. Large amount of bromophenols could be absorbed by the fish fed with the fish feed containing 30% *Sargassum siliquastrum* that the effect of feeding this feed on the bromophenol contents of the fish flesh would be more significant.

The increase in the bromophenol contents in the fish could affect the flavor of the flesh as the concentrations of 2-BP, 2,4-DBP and 2,4,6-TBP were 3.27, 4.66 and 28.9 *n*g/g fresh weight, respectively. These concentrations were greater than the threshold values which were $3x10^{-2}$ *n*g/g for 2-BP, 4 *n*g/g for 2,4-DBP and $6x10^{-1}$

*n*g/g for 2,4,6-TBP in water(Whitfield *et al.*, 1988). As 2,6-DBP was not detected in the fish flesh, iodine-like off-flavor would not occur. In order to produce the sea-like and ocean-like flavor, the concentrations of 2,4,6-TBP should be higher than 10 *n*g/g. The concentration of 2,4,6-TBP in the flesh of fish fed with the modified fish feed containing 30% *Sargassum siliquastrum* for eight weeks was 28.9 *n*g/g fresh weight. At these levels the bromophenols could affect the overall flavor of the fish flesh giving a sea-like or ocean-like flavor.

Besides, it was found that 2,4-DBP and 2,4,6-TBP were more abundant in the fish samples. This result was consistent with other seafood sample analyzed. The phenomenon could be explained by the amount of the bromophenols accumulated in flesh. The octanol/water partition coefficients (Log P, Table in chapter 2) of 2,4-DBP and 2,4,6-TBP were 3.00 and 3.74 respectively. According to Poels *et al.* (1988), compounds with Log P≥3.0 would have some bioconcentrations in the marine animal. Therefore, these two bromophenols were likely to accumulate in the flesh of the animal. As the other three bromophenols have Log P <3.0, the occurrence of such accumulation was lower. As a result, 2,4-DBP and 2,4,6-TBP were often the most abundant ones in most of the seafood and fish samples.

5.3.4 Sensory evaluation on the flesh of the fish fed with different fish feeds

Results of the Triangle tests carried out to evaluate the difference between the flavors of the fish flesh cultivated with the traditional feed and those with the modified feeds containing 30% *Padina arborescens* or 30% *Sargassum siliquastrum* showed the actual effects of the modified fish feeds on the overall flavor of the fish flesh. Each of the subjects was asked to perform four Triangle tests. Two of the tests evaluated the difference in the overall flavor between the control group and the experimental group fed with 30% *Padina arborescens* feed. Similarly, the other two were to evaluate between the control group and the experimental group fed with feed containing 30% *Sargassum siliquastrum*.

The subjects were asked to differentiate the odd one among the samples. Results were evaluated by the number of correct answers obtained and the total number of responses. Minimum number of correct answers were required to signify a difference between samples at 5% confident level (Roessler *et al.*, 1978; Meilgaard *et al.*, 1991). The results of the triangle tests are shown in Table 5.13 (p.122).

The total number of the responses was 44 for each type of fish fed with each type of modified feeds. In the evaluation of the flesh of the fish fed with modified

Table 5.13. Triangle tests results of the fish flesh fed with different fish feeds.

	Pad[a]		Sar[b]	
Subject	**Correct**	**Incorrect**	**Correct**	**Incorrect**
1	1	1	1	1
2	2	0	1	1
3	0	2	2	0
4	0	2	1	1
5	0	2	1	1
6	2	0	2	0
7	1	1	1	1
8	1	1	1	1
9	0	2	1	1
10	2	0	1	1
11	0	2	1	1
12	2	0	1	1
13	0	2	1	1
14	2	0	1	1
15	0	2	1	1
16	0	2	0	2
17	1	1	1	1
18	0	2	1	1
19	2	0	2	0
20	0	2	2	0
21	1	1	0	2
22	0	2	1	1
Total	**17**	**27**	**24**	**20**
Sig[c]	-		*	

[a]Evaluation on the meat of fishes fed with fish feed containing 30% *Padina arborescens* and that of traditional fish feed. No. of sets for each subjects was 2.

[b]Evaluation on the meat of fishes fed with fish feed containing 30% *Sargassum siliquastrum* and that of traditional fish feed. No. of sets for each subjects was 2.

[c]*Significant difference in flavor between the groups ($p<0.05$),
- no significant difference in flavor between groups ($p>0.05$)

feed containing 30% *Padina arborescens*, the number of correct answers was 17, which was lower than 22, the minimum number of correct responses. Thus, the difference in the flavor between the control group and the experimental group fed with modified feed mixed with 30% *Padina arborescens* for eight weeks was not significant ($p>0.05$).

In the tests on the flesh of the fish fed with feed containing 30% *Sargassum siliquastrum*, the number of correct responses was 24, which was higher than 21, the minimum required. Therefore, the difference between the flavors of the flesh from the fish fed with traditional feed and modified feed mixed with 30% *Sargassum siliquastrum* for eight weeks was significant at 5% confident level.

Besides, comments were obtained from the subjects. Some of them noted that the difference between the samples evaluating the flesh from the control group and the 30% *Sargassum siliquastrum* group was obvious. They noted that the flesh from the experimental group provided relatively stronger "sweet" and "seafood-like" flavors. The flavor was more characteristic.

The bromophenol contents in the flesh of fish fed with modified fish feed

containing 30% *Sargassum siliquastrum* were increased to levels sufficient to provide desirable sea-like flavor (2,4,6-TBP concentration > 10 ng/g fresh weight). Thus, subjects in the sensory test could differentiate it from the control and perceive "sweet" and seafood-like flavor in the fish flesh from the experimental group. It can be conclude that the effect of the feed with 30% *Sargassum siliquastrum* on the flavor of aquacultured fish was significant according to the increase in TBC and the sensory evaluation. The flavor quality could be affected by feeding different diets containing different amounts of bromophenols.

5.3.5 Growth of the fish fed with different fish feeds

It will be important to evaluate the effect of feeding different diets on the growth of fish. The growth of fish was represented by their weight change during the experiment [Table 5.14 (p.125)]. According to the statistical test results (two-way ANOVA), the weight of fish from the two experimental groups (fed with feed containing 30% *Padina arborescens* and 30% *Sargassum siliquastrum*) was not significantly different from that of the control group after eight weeks (p>0.05). Feeding modified feeds (contained seaweed) to fish did not significantly affect their weight because marine algae were thought to be potential a source of both protein and lipid for fish (Wahbeh, 1997). Thus, it is possible to incorporate marine algae

Table 5.14. Weight of the fish fed with different fish feeds.

	Weight of fish (g)		
	Group		
Week	Traditional Feed	Feed containing 30% *Padina arborescens*	Feed containing 30% *Sargassum siliquastrum*
0	94.0[a] ± 8.16[b]	94.0 ± 8.16	94.0 ± 8.16
2	102 ± 14	102 ± 8	99.1 ± 10.5
4	110 ± 6	96.9 ± 3.3	98.7 ± 6.0
6	117 ± 6	104 ± 2	119 ± 12
8	127 ± 15	121 ± 4	128 ± 7

Statistical test (two-way ANOVA)[c]

Source of Variation	***p*-value**	**Sig[d]**	***p*-value**	**Sig[d]**
Between Groups (Control and Experimental)	0.066	-	0.537	-
Interaction (Group × Time)	0.605	-	0.752	-

[a]Average weight of fish (g)

[b]Standard deviation of the weight of fish (g)

[c]Statistical test was performed to compare the weight of fish from the control group (Traditional feed) and the experimental groups (modified feeds).

[d] - no statistically significant difference ($p>0.05$) among samples

into fish feed to increase the bromophenol content in the aquacultured fish without affecting their growth.

5.4 Conclusion

The bromophenol concentrations detected in the aquacultured fish, *Siganus canaliculatus*, grown in Hong Kong were significantly lower than that of the wild-harvested one. The low bromophenol concentrations in aquacultued animals were believed to cause the lack of sea-like flavor in them. Methods were tested and developed to enhance the concentrations of bromophenols by increasing the bromophenol content in their diet. Marine algae were used as the sources of such compounds. Three types of modified fish feeds were developed including (1) fish feed prepared by mixing 10% *Padina arborescens* with 90% traditional fish feed powder; (2) fish feed containing 30% *Padina arborescens* and 70% traditional fish feed powder; and (3) fish feed containing 30% *Sargassum siliquastrum* and 70% traditional fish feed powder. All of the three types of the modified feeds possessed significantly higher bromophenol concentrations than that of the traditional feed ($p<0.05$). Feeds developed were used to grow the fish, *Sparus sarba*. Changes in the bromophenol concentrations were investigated at different times. Only the feed containing 30% *Sargassum siliquastrum* could significantly increase the TBCs in the

experimental fish after eight weeks ($p<0.05$). Sensory evaluations on the flesh from the experimental fishes were carried out. The flesh from fish fed with 30% *Sargassum siliquastrum* feed had significant difference in flavor compared with that fed with traditional feed ($p<0.05$). In short, this modified fish feed can increase the bromophenol contents of the experimental fish.

Chapter 6

General conclusion and significance of the study

In this study, the distribution and the seasonal variations of the bromophenols in selected seafoods and seaweeds found in Hong Kong were studied. Methods were tested to enhance the bromophenol contents in the cultured marine fish and subsequently to improve their flavor quality.

Bromophenols were detected in all seafood samples from marine sources including two marine fishes, two mollusks and two crustaceans found in Hong Kong. The concentrations of the bromophenols varied among and within each species at different time period in a year. The bromophenol contents in crab were generally the highest among all samples at 2420 ng/g. The concentrations of the bromophenols in most of the seafood samples were enough to give the distinct sea-like or brine-like flavor. TBCs detected in the gut of the marine fishes and the shrimps were higher than those in their flesh. Thus, bromophenols in the animals seemed to be obtained from their diets. None of the bromophenols were detected in the freshwater fish sample investigated.

As it was shown that bromophenols in the animals were obtained from their diet, it would be necessary to identify their possible dietary sources. Marine brown algae, which are capable of producing bromophenols, were considered to be the prime sources (Whitfield et al., 1997b; 1999). Three species of marine algae including *Padina arborescens*, *Sargassum siliquastrum* and *Lobophora variegata* found in Hong Kong waters were studied on their bromophenol contents. Bromophenols were found in all the marine algae samples. Their concentrations in the samples were relatively high. *Lobophora variegata* generally possessed the highest bromophenol content among the samples with the highest total bromophenol content detected at 7030 *n*g/g. Seasonal variations of the bromophenol contents in both marine animals and seaweed samples showed similar patterns with high concentration found in winter but low in summer in Hong Kong.

Marine algae containing abundant amount of bromophenols were considered to be sources of bromophenols found in other marine animals. Algae could be utilized in the aquaculture fishery to increase the bromophenol content of the products as the aquacultured fish contained low bromophenol concentrations and lacked the sea-like and marine-like flavor. To improve the flavor quality of the aquacultured fish, the fish feeds containing high bromophenol content were developed by incorporation of

marine algae into the feeds. The modified feeds produced were used to feed the marine fish, *Sparus sarba*. It was found that fish feed containing 30% *Sargassum siliquastrum* could significantly increase the bromophenol concentrations of the experimental fish after eight weeks. The increased concentrations of bromophenols in the flesh of fish fed with the modified fish feed containing 30% *Sargassum siliquastrum* were sufficient to produce sea-like flavor. Sensory evaluations showed that the flesh of the fish fed with the modified fish feed was significant different in flavor compared with that of the traditional feed ($p<0.05$). Thus, the modified fish feed containing marine algae could increase the bromophenol content and hence affect the flavor of the aquacultured marine fish.

This is a pioneer study about bromophenols in Hong Kong. This study provides information on the quantity of the bromophenols present in the marine organisms found in Hong Kong. Based on the test results, a method was established to improve the flavor quality of aquacultured marine fish by increasing its bromophenol contents. The utilization of marine algae in the feed used in the aquaculture fishery can be brought into the market after standard formulation has been made.

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