Journal of Hazardous Materials 215–216 (2012) 108–114

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Use of intestinal *Pseudomonas aeruginosa* in fish to detect the environmental pollutant benzo[*a*]pyrene

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ARTICLE INFO

Article history:
Received 12 November 2011
Received in revised form 14 February 2012
Accepted 15 February 2012
Available online 22 February 2012

Keywords:
Pseudomonas aeruginosa
Population
Benzo[*a*]pyrene
Indicator
Fish biomarkers

ABSTRACT

This study examined the potential of *Pseudomonas aeruginosa* abundance in the intestines of fish as an indicator of exposure to benzo[*a*]pyrene (BaP). *P. aeruginosa* populations were enumerated in juvenile African catfish (*Clarias gariepinus*) injected intramuscularly three days previous with 0, 10, 30, 40, 50 or 70 mg/kg of BaP. Hepatic EROD and GST activities and biliary fluorescent aromatic compounds (FACs) 1-OH BaP, 3-OH BaP, 7,8-D BaP and BaP were quantified to investigate agreements between the new indicator and established fish biomarkers. The shape of bacterial population (logarithm of colony-forming unit) dose-response curve generally matched those of biliary FACs concentrations. Conversely, the EROD and GST dose-response curves were generally the mirror images of the bacterial population curve. Changes in intestinal *P. aeruginosa* population appear to be an indirect effect of BaP exposure because exposure to 0–100 μg/ml BaP had no effect on *P. aeruginosa* populations grown on agar plates containing BaP. Using intestinal *P. aeruginosa* population of fish as a universal indicator of BaP pollution in aquatic environments is discussed.Conversely, the EROD and GST dose-response curves were generally the mirror images of the bacterial population curve.

1. Introduction

There is increasing interest in minimizing anthropogenic impacts on water bodies and associated biota [1]. Physical and chemical analyses of environmental compartments provide some of the monitoring information required but are unable to determine the bioavailability of environmental pollutants. Therefore, "bioindicators" and "biomarkers" in fish and other aquatic biota have been used in environmental risk, and impact, assessments. Individual biomarkers seldom provide all information required [2] so typically a suite of biomarkers is preferred [3–5] which may raise issues of cost [6]. For these reasons there is continuing interest in the development of new, easily measured, sensitive and cost-effective indicators of environmental stress [7].

Microbial degradation is one of the main routes through which pollutants are ultimately eliminated in the environment [8]. However, little attention has been paid in monitoring bacteria as potential indicators of aquatic pollution. Research that has been carried out has focused on the measuring bacteria in the environment [9–12] with only limited studies investigating endogenous bacterial abundances found within aquatic animals [13–16]. Furthermore, effects of persistent organic pollutants (POPs) on endogenous bacteria of organisms have rarely been investigated and we know no study which has related endogenous bacterial responses to other biomarkers in fish exposed to xenobiotics.

Pseudomonas is a waterborne, Gram negative, rod-shaped genus of bacteria that is tolerant to a wide range of xenobiotics, including PAHs [17–19], and plays an important role in the treatment of petroleum contaminated lands [20]. Diverse metabolic activities enable *Pseudomonas* bacteria to metabolize various substrates and colonize niches [21] as diverse as natural mineral waters [22] and ice crystal cores in clouds [23,24]. *Pseudomonas aeruginosa* is an opportunistic human pathogen [25] but has been used as a probiotic in aquaculture [26,27]. Sivakami et al. [28] found *P. aeruginosa*, along with *Escherichia coli*, to be the dominant bacteria in intestinal tracts of Indian carp (*Catla catla*), Rohu (*Labeo rohita*),

Abbreviations: 7,8-D BaP, 7,8dihydrodiolbenzo[*a*]pyrene; 1-OH BaP, 1-hydroxybenzo[*a*]pyrene; 3-OH BaP, 3-hydroxybenzo[*a*]pyrene; EROD, ethoxyresorufin-*O*-deethylase; GST, glutathione-*S*-transferase; FACs, fluorescent aromatic compounds.

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doi:10.1016/j.jhazmat.2012.02.038

Mrigal (*Cirrhinus mrigala*) and Common carp (*Cyprinus carpio* L.). *P. aeruginosa* is able to grow in a highly polluted environment with polycyclic aromatic hydrocarbons (PAHs) and crude oils [29,30]. Benzo[*a*]pyrene (BaP) is a carcinogenic, teratogenic and mutagenic five-ringed PAH [31,32] and is widespread in aquatic environments [33]. BaP has been used as a model in many toxicological studies [31]. It can be degraded by bacteria [34,35] including bacteria of the *Pseudomonas* genus; Juhasz et al. [36] reported 20–30% reduction in BaP concentration in a basal salt medium after 63 days of incubation with three strains of *P. cepacia*.

In this study we chose African catfish (*Clarias gariepinus*) because of its widespread distribution in tropical and subtropical regions and its resistance to adverse environmental conditions [37–39]. The main objective of this study was to investigate the potential of intestinal *P. aeruginosa* population as a new indicator of environmental pollution. In addition to quantifying intestinal *P. aeruginosa* numbers in response to graded doses of BaP injected intramuscularly, we also compared this response to that of established fish biomarkers: hepatic ethoxyresorufin-*O*-deethylase (EROD) and glutathione *S*-transferase (GST) activities, and selected biliary concentrations of selected fluorescent aromatic compounds (FACs). Finally, we exposed *P. aeruginosa* directly to graded doses of BaP on Cetrimide Agar plates to determine whether the intestinal bacterial population changes were due to direct effects of BaP, or rather reflected physiological responses of the fish.

2. Materials and methods

2.1. Chemicals

7,8-dihydrodiolbenzo[*a*]pyrene (7,8-D BaP), 1-hydroxybenzo[*a*]pyrene (1-OH BaP) and 3-hydroxybenzo[*a*]pyrene (3-OH BaP) high performance liquid chromatography (HPLC) standards were purchased from the Mid-west Research Institute (Kansas City, MO, USA); β-glucuronidase/arylsulfatase (from *Helix pomatia*) and Cetrimide agar from Merck (Germany); bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol (DTT), BaP, glutathione (GSH), 7-ethoxyresorufin (7-ER), nicotinamide adenine dinucleotide phosphate (NADPH) from Sigma Chemical (St. Louis, USA); Resorufin sodium salt from Sigma–Aldrich (USA); and low-binding protein membrane filters (0.45 μm) and sterile Acrodisc® syringe filters (0.2 μm) were obtained from Pall Life Science (USA).

2.2. Experimental design

2.2.1. Fish

Fish were bred in Puchong's Aquaculture Centre and reared for three months in 1.8 m diameter circular fiberglass tanks filled with 700 L water. At the time of the experiment fish average weight was 131(±6.3) g and all fish were immature. Fish were fasted 24 h before commencement of the experiments.

2.2.2. Organ/substance selection

A pre-experiment was conducted to determine which organ or substance within *C. gariepinus* showed highest numbers of *P. aeruginosa*, therefore most suitable to be used for further study. A few fish were killed by an overdose of clove oil (0.2 ml/L) and sampled for liver, bile, gills, skin mucous and intestinal materials. Each of them was macerated with an autoclaved mortar and pestle and 0.5 g was mixed with 4.5 ml of normal saline. Five volumes of diluents (normal saline) were added to obtain final concentrations of 10^{-1} to 10^{-5}. Subsamples of 0.1 ml from each dilution were plated on Cetrimide Agar and incubated at 36 °C for 48 h producing colony counts between 30 and 300. Data were presented as logarithm of colony-forming units per g of the used organ/substance (log CFU/g). The organ or substance with the highest *P. aeruginosa* population (intestinal material) was selected as the target for the bacterial sampling during this study.

2.2.3. Exposure to BaP and sampling

African catfish were tagged with anchor bar tags for individual identification and intramuscularly (i.m.) injected with 0, 10, 30, 40, 50 or 70 mg/kg of BaP dissolved in corn oil as the carrier. Fifteen fish were injected with each BaP dose to provide three 3 replicate pools of 5 fish each. Thereafter, fish were randomly distributed among three 1.8 m diameter circular fiberglass tanks filled with 700 L water. The highest dosage of BaP (70 mg/kg) killed one fish in each of the three replicates 24–48 h post-injection. These three fish were moved to a separate tank as soon as distress was evident to avoid cannibalism by tank-mates [38] but none of them survived to be sampled on day 3. Water in tanks was continuously filtered (1250 L/h) through active carbon bags to absorb any BaP leaked from injection points. Three days later fish were killed by an overdose of clove oil. To minimize contamination of organs to be sampled the fish body surface was immediately washed three times with 70% ethanol and then three times with sterile distilled water. Gall bladders and livers were dissected aseptically, snap-frozen in liquid nitrogen and stored at −80 °C until analysis. EROD and GST activities and biliary FACs quantifications were carried out as described in Karami et al. [40]. Briefly, livers of the five fish within each replicate were pooled, homogenized and then assayed for EROD activity based on the method of Hodson et al. [41] modified for microplate assay by Frasco and Guilhermino [42] with some modifications using 7-ethoxyresorufin as the substrate and NADPH as the cofactor. Similarly, GST activities were quantified by the method of Frasco and Guilhermino [42] using CDNB and GSH as the substrates. To quantify biliary FACs, whole bile samples of the five fish were pooled, mixed by vortexing and subsamples were hydrolyzed, followed by the two-step bile preparation protocol [43] and quantified as described earlier [40] through an Agilent 1200 series HPLC (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Each assay was repeated twice per replicate and the mean of the two replications was carried forward for statistical analysis.

2.2.4. Bacteriological studies

2.2.4.1. Intestinal P. aeruginosa enumeration. Populations of intestinal *P. aeruginosa* were estimated based on the dilution plate technique. Entire intestines (from stomach to anus) of the five fish in a replicate were dissected, contents were stripped out with sterile forceps, pooled, and macerated using an autoclaved mortar and pestle. After making dilutions, subsamples were plated as described in Section 2.2.2.

2.2.4.2. Pseudomonas aeruginosa identification. P. aeruginosa colonies were confirmed through cultural characteristics including production of blue, blue–green, green or yellow–green fluorescence on Cetrimide Agar under UV light (360 ± 20 nm) [44], growth at 42 °C, and not growing at 4 °C [45,46].

2.2.4.3. Plate experiment. To investigate the direct effects of BaP on *P. aeruginosa* abundance, different quantities of BaP were dissolved in ethanol, filtered through a sterile syringe filter (0.2 μm) to remove any spores or bacteria and then added into Cetrimide Agar to produce final concentrations of 0, 10, 25, 50 and 100 μg/ml Cetrimide Agar corresponding to the range of total quantified biliary FACs and higher. *P. aeruginosa* subsamples were plated on the Cetrimide Agar (three replicate plates for each BaP concentration), and incubated at 36 °C for 48 h before colonies were quantified.

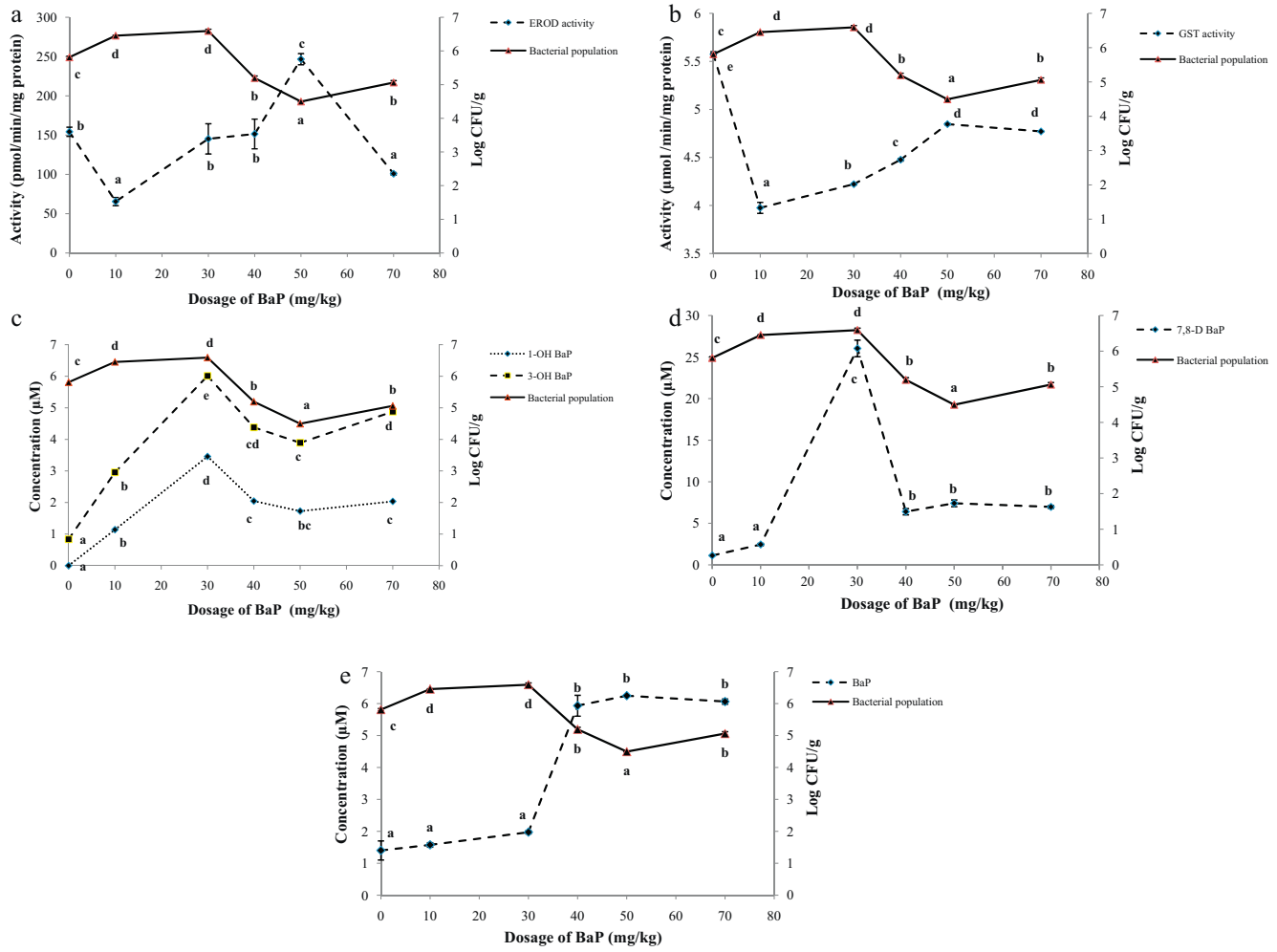

Fig. 1. Dose-response curves for *Clarias gariepinus* exposed to intramuscular injection of BaP showing relationships between intestinal *Pseudomonas aeruginosa* counts and (a) EROD activities (b) GST activities (c) 1-OH BaP and 3-OH BaP concentrations and (d) 7,8-D BaP concentrations and e) BaP concentrations. Each point is the mean (±SE) of 3 replicates; Points with different letters within each parameter are significantly different (Duncan's multiple range tests, $P \leq 0.05$).

2.3. Statistics

Prior to statistical analyses, all ANOVA and MANOVA assumptions were tested [47]. Separate ANOVAs were performed to examine the influence of dosage of injection on each biliary FAC, and bacterial population (intestinal and plate counted). A MANOVA was run to test the influence of dosage of injection on EROD and GST activities. Post hoc Duncan's multiple range tests were run when significant differences were detected. All statistical tests were performed through Predictive Analytics software (V. 18, SPSS Inc., USA) except for descriptive statistics which were calculated by Statistix software (V. 8, 2007 Analytical Software, USA).

3. Results

Liver, bile, gills and skin mucous produced negligible (<30 CFU) counts of *P. aeruginosa*. In contrast, intestinal materials produced the highest number of the bacterial counts. Therefore, all further work was done with intestine.

Injection of different BaP dosages significantly ($P \leq 0.001$) influenced all biomarkers quantified and also intestinal *P. aeruginosa* populations. Fig. 1a and b show the generally inverse negative relationship between intestinal *P. aeruginosa* population changes and EROD and GST activities, respectively. The lowest dose of BaP injected (10 mg/kg) increased *P. aeruginosa* numbers but decreased EROD and GST activities. *P. aeruginosa* count then remained unchanged from 10 to 30 mg/kg. Bacterial population decreased from 30 to 50 mg/kg BaP while EROD and GST activities increased. The highest dose applied (70 mg/kg) decreased EROD activity while *P. aeruginosa* counts recovered somewhat.

The dose-response relationships of the biliary phenolic metabolites (1-OH BaP and 3-OH BaP) were generally similar in shape to the *P. aeruginosa* dose-response curve, increasing to 30 mg/kg, then decreasing to 50 mg/kg before recovering slightly at 70 mg/kg (Fig. 1c). 7,8-D BaP generally followed this pattern as well (Fig. 1d) without the slight rise in response to 70 mg/kg. Low concentrations of biliary BaP (0–30 mg/kg) were associated with elevated *P. aeruginosa* counts whereas higher concentrations of biliary BaP (30–70 mg/kg) were associated with depressed *P. aeruginosa* counts (Fig. 1e).

Concentrations of BaP in Cetrimide Agar, ranging from 0 to 100 µg/ml, had no significant effect on *P. aeruginosa* CFU counts after 2d incubation at 36 °C (Fig. 2).

4. Discussion

Survival of bacteria in the natural environment is restricted by many ecological and biological factors such as solar radiation, oceanographic factors and bioavailability of organic compounds

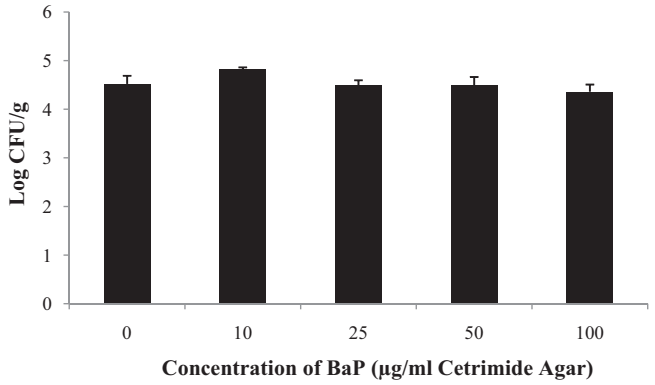

Fig. 2. *Pseudomonas aeruginosa* population (log CFU/g) plated on Cetrimide Agar containing different concentrations of BaP; $n = 3$.

[48–50]. Fish may harbor higher numbers of bacteria than their surrounding environment [51] making them more appropriate for microfloral sampling than sediment or water. By developing parsimonious three-trophic level community model, Arkoosh et al. [52] suggested more sensitivity and more reliability of using salmon (*Oncorhynchus* spp.) parasites than salmon themselves in monitoring contaminants. In another study, fish macro-parasite assemblages were suggested as sensitive indicators of environmental stressors, such as pollutants, because they exhibit the overall interactions of their host with other members in a community [53]. Within fish the intestines has been recommended by the Furunculosis Committee for isolating bacteria [54] which is consistent with our finding of higher *P. aeruginosa* numbers in the intestines than other tested organs and substances.

To our knowledge this study is the first to investigate the potential of intestinal bacteria to serve as a biomarker of exposure to an organic pollutant in fish. We were able to find only one study which examined changes in the population of endogenous bacteria (symbiotic sub-cuticular bacteria) in an aquatic organism (echinoderms) and this was in response to oil pollution [55] with no supporting results from biomarker studies.

Bacteria associated with marine sponges have been examined as a potential indicator of environmental pollution in a few studies [e.g., 13,56]. High filtration rates result in marine sponges accumulating bacteria which have been exposed to the surrounding environment. Kefalas et al. [13] proposed quantification of certain bacteria found within *Spongia officinalis* as an indicator of coastal marine pollution. However, responses of intestinal bacteria in fish may be quite different from the population of bacteria in sponges. Bacteria from the egg or food and water colonize the fish intestine in early life stage [57] and are established after metamorphosis or during the juvenile stage [14]. After early life stages bacterial populations can be influenced by ingested food and water [58,59] but also could be influenced by physiological parameters. For example, complex interactions between the gut-associated lymphoid tissues (GALT), which defend against infectious agents and regulate immunity in the alimentary tract, and gasterointestinal bacteria are indicated in many studies [e.g., 60,61]. A recent study reported GALT apoptosis in rainbow trout (*Oncorhynchus mykiss*) exposed to environmental antigens [62]. Therefore, immunological responses to BaP in the present study could be implicated in the observed changes to intestinal flora, as well as other physiological parameters such as endocrine responses [63,64]. Some studies have reported reduced fish resistance against bacterial challenges after exposure to PAHs [65,66]. Bacterial proliferation has been shown in mucus of Japanese flounder (*Paralichthys olivaceus*) exposed to heavy oil, possibly due to decrease in leukocyte titer [67]. Lab studies have examined intestinal bacterial changes in fish [15] and horse leeches [16] exposed to heavy metals in food or water. Changes observed in these studies could result from direct effects of the metals on the bacteria or indirect effects mediated through the host. In the present study we attempted to distinguish between these two routes of effect by exposing *P. aeruginosa* to graded doses of BaP directly on agar plates. The lack of change in bacterial counts on the plates indicates that the mechanism through which *P. aeruginosa* populations changed in the intestines of African catfish reflected physiological responses of the fish to BaP. Furthermore, in order to minimize direct contact of BaP with intestinal bacteria in the present study we chose i.m. injection over intraperitoneal (i.p.) injection.

Previous work in our lab has shown that i.m. injection of BaP results in lower accumulation of biliary FACs compared to i.p. injection [40]. Therefore, to ensure sufficient physiological responses within the fish, and also to test the bacterial responses in a highly impacted environment, dosages of 50 and 70 mg/kg were also examined in this study. Bacterial responses to high BaP exposure may suggest application of this measure to outright releases of PAHs into the aquatic environment. Results demonstrated that agreement between the established biomarkers and the bacterial population changes is not limited by dosage of injection.

Bacterial population increases in response to 10 and 30 mg/kg BaP may be explained by the hormesis phenomenon [68]. Higher doses of BaP reduced bacterial counts with modest recovery at the highest concentration of 70 mg/kg. The latter might be related to the availability of organic compounds provided by dead bacteria. Generally, the trend of *P. aeruginosa* population changes was negatively related to the EROD and GST activities. Complex cross talks between biotransformation enzymes and immune systems have been documented in fish [69]. Our findings are to some degree consistent with the suppression of GST and EROD activities in 3-methylcholanthrene (3-MC) exposed carp (*C. carpio*) after infection with *Listeria monocytogenes* (a facultative anaerobe bacterium) [70]. In another study lipopolysaccharide (LPS), an immunostimulant which mimics bacterial infection effects, injection inhibited 3-MC-induced cytochrome P450 and GST activities in the liver and head kidney of carp [71]. In some situations, established biomarkers may become undetectable [e.g., 40,72,73]. Therefore, in future studies it could be possible to replace the undetectable biomarkers with the new indicator (intestinal *P. aeruginosa* population).

In the present study the bacterial population dose-response curves generally matched those of the four biliary BaP FACs 1-OH BaP, 3-OH BaP, 7,8-D BaP, and BaP. Since feeding ceased 24 h before the experiment bile should have been retained in the gall bladder preventing bacterial contact with the BaP metabolites. More likely is that injected BaP produced some physiological changes in the fish which in turn altered intestinal bacterial populations and biliary metabolites. Various studies in mammals and fish have shown BaP to be immunusuppresive following biotransformation [e.g., 74,75]. For example, BaP caused immunosuppression through cytochrome P4501A dependent production of immunotoxic BaP metabolites in Japanese medaka (*Oryzias latipes*) [76]. In another study reduction of BaP biotransformation to 7,8-D BaP through administration of α-naphthoflavon alleviated immunotoxicity [77]. Further research will be required to identify potential cross talks between phase I and II enzymes, biliary FACs and intestinal bacteria.

Many factors may obscure links between environmental stressors and biological responses. For example in river ecosystems factors including physical alteration of the river, climate change, species interactions and food abundance can affect biological responses [78]. Similarly, it is likely that the population of intestinal bacteria is influenced by factors other than environmental pollutants. For example Ringø et al. [79] showed important influences of salinity, antibiotics, chromic oxide, diet and dietary components on bacterial abundance in the salmonid intestine. Geldreich and Clarke

[51] showed effects of food availability, as well as water contaminant levels, on the intestinal bacterial abundance of 14 different freshwater fish from Little Miami River. However, *P. aeruginsa* has shown the ability to withstand high concentrations of salts [80], dyes [81] and some antiseptics [82]. Furthermore, *P. aeruginosas* should not be affected by a wide variety of antibiotics because it has been demonstrated to broadly resistant [83,84]. In addition, current concentrations of antibiotics in contaminated aquatic environments are well below concentrations shown to inhibit *P. aeruginosa* abundance [e.g., 85,86]. *Pseudomonas* is one of the dominant genera of gastrointestinal bacteria in freshwater and marine fish [28,87,88], possibly because of a wide range of metabolic activities and also because it produces substances which inhibit the growth of other bacterial species. For example *P. aeruginosa* inhibited the growth of *E. coli* in intestine of *Gambusia affinis* [89]. All of these features give credit to the application of *P. aeruginosa* population in various fish species and different environments.

Whether numbers of intestinal *P. aeruginosa* population should be termed a biomarker or a bioindicator is an interesting question. On the one hand *P. aeruginosa* population may not be considered as a biomarker because, rather than being part of a physiological reaction of the fish, they are simpler independent biological systems inside a more complicated host organism. On the other hand, bioindicators are defined as organisms which provide information on habitat condition by their presence, absence or behavior [90]. As such, intestinal *P. aeruginosa* population may not be considered a bioindicator because our results suggest they are not directly exposed to, or reacting to, pollutants but rather are reflecting pollutant responses of the fish in which they live. Therefore, endogenous fish micoflora (including bacteria, viruses and parasites) may constitute a separate group of environmental pollutant indicators other than “biomarkers” or “bioindicators”.

Quantifying *P. aeruginosa* population as an environmental indicator is inexpensive, quick, easy and requires only basic laboratory equipment. Therefore, the current study showed that environments could be monitored by caging fish of known history for several days without feeding. Such a program would also benefit from testing of water samples for background levels of *P. aeruginosa* population. Furthermore, there may be ways to circumvent one of the main drawbacks associated with measurement of many biomarkers in fish which is the need for destructive sampling. It may be possible to quantify *P. aeruginosa* from faecal samples.

Similar to the other environmental indicators, further studies could be conducted in order to identify influence of populations, ages, sexes, times of year, and reproductive states on the intestinal bacterial population. Taken together, we conclude that intestinal *P. aeruginosa* population in different fish species may be used as a universal indicator of BaP pollution. However, quantification of intestinal *P. aeruginosa* population merits further research as an indicator of environmental pollutant impact on fish.

5. Conclusions

1. Intestinal *P. aeruginosa* population is a reliable, easily measured and cost-effective indicator of environmental pollutant benzo[*a*]pyrene.
2. Intestinal *P. aeruginosa* population changes show agreement with other established biomarkers but may, in some circumstances, be more sensitive.
3. Intestinal *P. aeruginosa* population may be considered as a universal indicator of BaP pollution in aquatic environments.
4. Endogenous microflora may constitute a new group of environmental pollutant indicators.

Acknowledgments

Special thanks must go to Professor Tracy Collier for his helpful comments on the final draft of this manuscript and to Professor Peter Hodson for his useful thoughts during the early stages of this study. Thanks are extended to Professor Mad Nasir Shamsudin, Professor Mohamad Pauzi Zakaria, Professor Ahmad Makmom b. Hj Abdullah, Mr. Jasni bin md. Yusoff, Mr. Tengku Shahrul Tengku Md. Yusoff, Mrs. NoorAzlina bt. Masdor, Mrs. Noorshinah bt. Hussin and Mr. Amin Jokar for their helpful assistance during running the experiments.

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