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The Bacteria, Proximate Composition, and Polycyclic Aromatic Hydrocarbon Content of Smoked Catfish (*Clarias gariepinus*)

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ABSTRACT The microbiological quality, proximate composition and Polycyclic Aromatic Hydrocarbon content of smoked catfish (*Clarias gariepinus*) Yenagoa and Port Harcourt metropolis was investigated. Smoked catfish samples were purchased from five different markets (Igbogene, Tombia, Opolo, Swale, and Rumuokoro). The bacteria associated with the fish samples were determined using standard plate count methods with the aid of multipurpose and selective media. Moisture content, crude protein, total fat, crude fibre and ash were analyzed with the method A.O.A.C. The concentrations and composition of Polycyclic Aromatic Hydrocarbons (PAHs) was achieved using the principle of gas chromatography with flame ionization. The highest and lowest viable count of heterotrophs of 5.4 x 10^6cfu/g and 4.0 x 10^5cfu/g was obtained in the smoked catfish samples from Igbogene and Tombia markets respectively. A total of seven bacteria genera were isolated from the smoked catfish samples. The species and their frequency of occurrence are; *Bacillus* sp (34%), *Enterobacter* sp (7%), *Escherichia coli* (13%), *Micrococcus* sp (7%), *Pseudomonas* sp (13%), *Salmonella* sp (13%), and *Shigella* species (13%). However, only two oil degraders *Bacillus* sp (71%) and *Pseudomonas* sp (29%) were isolated. The proximate analysis showed that moisture content ranged from 17.59 ± 0.21 to 9.84 ± 0.24%, the crude protein ranged from 35.18 ± 0.86 to 13.15 ± 0.78%, fat ranged from 27.92 ± 0.19 to 17.19 ± 0.48% and the ash content ranged from 8.35 ± 0.22 to 2.08 ± 0.00%, while crude fibre was not detected. A total of fifteen PAH was detected in the smoked catfish samples. The maximum and least total PAHs content of 45.57 μ g/kg and 18.33 μ g/kg was obtained from fish samples

from Igbogene and Opolo respectively. However, Benzo(a)anthracene was not detected in Igbogene catfish samples. The values of individual PAH in some of the samples were higher than the European Standard for individual PAHs level (5 μ g/kg) in foods. Benzo(a)pyrene a marker for the occurrence and effect of carcinogenic PAHs in food was detected in all the fish samples with values ranging from 0.10 μ g/kg in Swale catfish to 0.14 μ g/kg in Igbogene catfish samples. Flouranthene was available in all the fish samples with values ranging from 30.10 to 6.42 μ g/kg recording the highest PAH values. The lowest PAH values was recorded by anthracene (0.02 to 0.05 μ g/kg). However, acenaphthene and acenaphthylene were not detected in the fish samples. The occurrence of *E. coli* in catfish from some of the markets indicates feacal contamination which poses a serious health challenge to unsuspecting consumers. The observed presence of PAHs in smoked fish in Yenagoa and Port Harcourt metropolis calls for caution because this may pose health risk both to the handlers and consumers due to the carcinogenic nature of PAHs and its ability to bioaccumulate in the body of thereby predisposing the people to cancers.

Keywords: Clarias gariepinus, *E. coli*, PAH, Benzo(a)pyrene, bacteria, fungi, oil degraders

Introduction

Fish is a rich source of protein. It is a good source of lysine, thiamin, riboflavin, vitamins such as vitamins A and D and minerals such as phosphorous, calcium and iron. It is high in polyunsaturated fatty acids that are important in lowering blood cholesterol level (Al-Jedah *et al*., 1999). Fish constitute about 45% of the total amount of protein (FDF, 2007). The daily increase in demand for fish and fish products in Nigeria has led to increased fish production by both public and private sectors in order to meet this demand (Foraminifera, 2013). Fish is a perishable item that requires adequate preservative measures so that it can have a longer shelf-life (Abolagba and Melle, 2008). As soon as fish dies, it remains in first class quality only for a short while. However, spoilage soon sets in which is occasioned by an increase in the ambient temperature that triggers favourable conditions for microorganisms to thrive. This deterioration is due to growth of microorganisms or non-microbial causes such as lipid oxidation (Martin, 1994). Consequently, there is the necessity for smoking in order to preserve the quality of

fish. Smoked fish are fishes that have been cured by smoking. There are two general ways of smoking fish; this is hot smoking and cold smoking. Hot smoking is also called barbecuing and requires a short salting time and smoking temperature of about 32 ℃ for the first two hours and 66 ℃ for an additional four to eight hours. Smoking, apart from giving the product a desirable taste and odour provides a longer shelf-life through its anti-bacterial and oxidative effect, lowering of pH, imparting desirable colouration as well as accelerating the drying process and acting as antagonist to spoilage agents (Eyo, 2001; Sengor *et al*., 2004). Polycyclic aromatic hydrocarbons (PAHs) are formed as a result of incomplete combustion or thermal decomposition of the organic materials. Thus, the PAHs formed in the fish during the smoking process are as a result of the pyrolysis of the fats present in the fish (Phillips, 1999; Kazerouni *et al*., 2001). The Scientific Committee on Food concluded in its opinion of 4th December 2002 that a number of heavy PAHs are carcinogens and that Benzo(a)Pyrene can be used as a marker for the occurrence and effect of these carcinogenic PAHs in food (SCF, 2002). Conditions of smoke generation can dramatically influence the level of PAHs in smoked foods (Toth and Potthast, 1984). Rochelle *et al*., (2005) suggested that *Bacillus subtilis* is able to degrade high molecular weight PAH; its use in the clean -up of PAH-contaminated sites will go a long way in improving the health of the environment for the benefit of humankind.

In Bayelsa State, smoking is widely used for fish preservation. Smoke-drying is achieved using kilns with firewood as fuel for drying. After processing, the products are placed in locally made baskets or jute sacs ready for transportation to various markets within and outside the state. Often, the products are not properly handled, packaged and stored (Oku and Amakoromo, 2013). This study aims to determine the bacteria load and proximate composition of smoked catfish (*Clarias gariepinus*) as well as the associated microbes with bioremediation potentials; to determine the polycyclic aromatic hydrocarbons (PAHs) content consequently imparted to the smoked fish through emissions from wood fire.

Materials and Methods

Sample collection and preparation

Smoked catfish (*Clarias gariepinus*) samples of average size were purchased from five different major markets (Tombia, Rumuokoro, Opolo, Igboghene, and Swale markets respectively) located in Yenagoa metropolis in Bayelsa state. Each catfish samples were purchased from different smoked fish sellers within the markets. They were transported in ice-packed coolers to the laboratory and consequently refrigerated prior to analysis. Smoked fish sam-

ples were homogenized in a Marlex blender, packaged in medium sized plastic bowls and labeled appropriately for analyses.

Determination of bacteria load

Standard plate count method was used to determine the total microbial load making use of the sterile nutrient agar. Other agars employed for the determination of bacteria include MacConkey agar, Salmonella-Shigella agar and Eosin Methylene blue agar. Each agar was prepared according to the manufacturer's instructions. Mineral salt medium that contains a single hydrocarbon source (Bonny light crude oil) was used to determine bacteria with bioremediation potentials via vapour phase transfer. The mineral salt media was prepared with the following composition: K_2HPO_4 (1.8g/l); NH_4Cl (4g/l); $MgSO_4.7H_2O$ (0.2g/l); NaCl (0.1g/l); $NaSO_4.7H_2O$ (0.01g/l); agar-agar (20g/l); and distilled water (1L) and buffered to pH 7. All media were sterilized at in the autoclave at 121 °C for 15 minutes as described by Jyothi *et al*., 2012. Media were poured into sterile plates aseptically.

Isolation of Total Heterotrophic Bacteria from Smoked Catfish

One gram (1.0g) of finely homogenized fish sample was transferred aseptically into 9ml sterile distilled water in test tube giving a tenfold dilution (10^{-1}). 1ml was taken from this dilution into the next test tube containing 9ml of sterile distilled water to achieve 10^{-2} dilution. This sequence was repeated until dilution 10^{-6} was obtained. 1ml aliquot of 10^{-3} dilution was transferred aseptically unto sterile Petri dishes and 10ml of molten sterile agar cooled to about 45^{o}C was added aseptically, swirled and allowed to solidify. Samples were plated in duplicates. Plates were incubated in an inverted position at 37 ± 2^{o}C for 24 hours after which viable colonies were counted. Uninoculated plates served as control.

Isolation and Identification of Hydrocarbon Utilizing Bacteria via Vapour Phase Transfer

The mineral salt media was prepared as described by Jyothi *et al*., 2012. 1ml of the diluents from 10^{-3} dilution was transferred aseptically into sterile petri dish and 10ml of sterile mineral salt agar cooled to about 45^{o}C was added aseptically, swirled and allowed to solidify. Whatman filter paper of 99mm in diameter were sterilized in the oven, cooled, and soaked in crude oil aseptically. A sterile forceps was then used to place the soaked filter paper in the lid of the sterile petri-dish and then placed over the inoculated plates (Thijsse and van der Linden, 1961). Samples were plated in duplicates. Plates were

incubated in an inverted position at 37 ± 2°C for 7 days after which viable colonies were counted. Uninoculated plates served as control.
After the incubation periods, morphologically distinct and discrete colonies were streaked on agar plates to obtain pure cultures which were then stored in nutrient agar slants for further biochemical and characterization tests. These pure isolates were identified by the method described by Collins *et al.,* (1989) and Cheesebrough (2002).

Determination of Proximate Composition of Smoked Catfish

Parameters analyzed include moisture content, crude fibre, crude protein, total fat and ash. These were analyzed with the method A.O.A.C (1995).

Extraction of PAH from catfish samples and Gas Chromatographic analysis

Ten milliliters (10ml) of dichloromethane was added to 2 grams of finely homogenized fish sample, mixed thoroughly with glass rod and allowed to settle. The mixture was carefully filtered and the extract obtained concentrated to 2ml.

Sample clean up/separation: The concentrated extract was passed through 10mm, ID X 250mm column packed with glass wool, silica gel and anhydrous sodium sulphate. The extracted samples were passed through the column using 10ml 1+1 dichloromethane-acetone as an elution solvent. Each eluate was concentrated to 1ml prior to injection into gas chromatogram (GC Model HP 5890 series II GC-FID, Made in USA) for PAHs analysis.

Gas Chromatography analysis: One microlitre (1 μl) of the eluate was injected by means of a hypodermic syringe through a rubber septum in the column. The retention for the PAHs analysis lasted for 30 minutes. PAH components were automatically detected as they emerged from the column (at a constant flow rate) by Flame Ionization Detector whose response was dependent on the composition of the vapor, by measuring the detector time. Components of low solubility in the stationary phase took a shorter time to be transported through the column while components of high solubility in the stationary phase took a longer elution time thus leading to the differential motilities of the fractional components of the polycyclic aromatic hydrocarbons (USEPA 1996).

Results

Microbial analysis of the total mean count of viable bacteria of the various

groups of bacteria from smoked *Clarias gariepinus* from the various markets is presented in Table 1. The highest total heterotrophic bacterial load of 5.4 x 10^6 was recorded in catfish samples obtained from Igbogene market while the lowest bacterial load of 4.0 x 10^5 was obtained in Tombia samples.

Table 1: Bacteria Counts (cfu/g) of Smoked Clarias gariepinus from the Various Markets

Market	Counts of Bacteria (cfu/g)				
	Total heterotrophs	**Total oil degraders**	***E. coli***	**Total coliform**	***Salmonella Shigella***
Igbogene	5.4 x 10^6	1.5 x 10^4	1.70 x 10^6	1.0 x 10^6	5.4 x 10^6
Opolo	6.0 x 10^5	2.7 x 10^3		4.0 x 10^5	0
Rumuokoro	8.0 x 10^5	3.3 x 10^3	3.0 x 10^5	0	0
Tombia	4.0 x 10^5	1.4 x 10^3	1.0 x 10^6	0	0
Swale	1.4 x 10^6	1.0 x 10^3	0	0	0
			0		

Characterization and identification of the bacterial isolates showed that only species of seven bacterial genera occurred in the smoked catfish (*Clarias gariepinus*) samples. The species and their frequency of occurrence are; *Bacillus* sp (34%), *Enterobacter* sp (7%), *E. coli* (13%), *Micrococcus* sp (7%), *Pseudomonas* sp (13%), *Salmonella* sp (13%), and *Shigella* species (13%). On the other hand, only two oil degraders were isolated and they were *Bacillus* sp (71%) and *Pseudomonas* sp (29%).

The Proximate composition (%) of smoked catfish samples is shown in Figure 1. The mean and standard error of moisture content ranged from 17.59 ± 0.21 to 9.84 ± 0.24%, the crude protein ranged from 35.18 ± 0.86 to 13.15 ± 0.78%, fat ranged from 27.92 ± 0.19 to 17.19 ± 0.48% and the ash content ranged from 8.35 ± 0.22 to 2.08 ± 0.00%, while crude fibre was not detected.

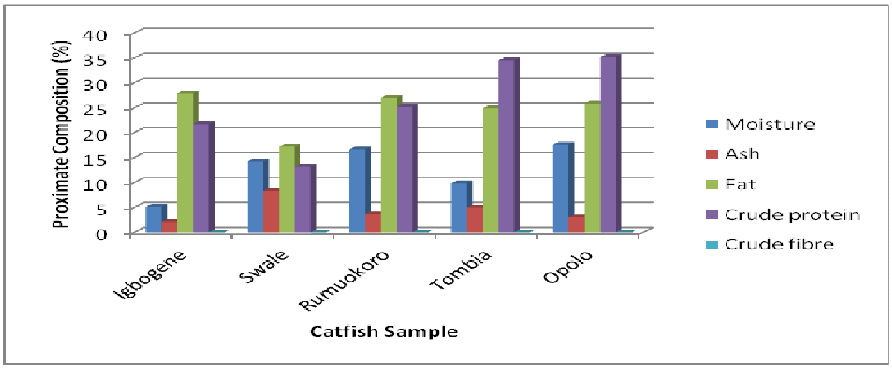

Fig. 1: Proximate composition (%) of Smoked Catfish (Clarias gariepinus) Samples

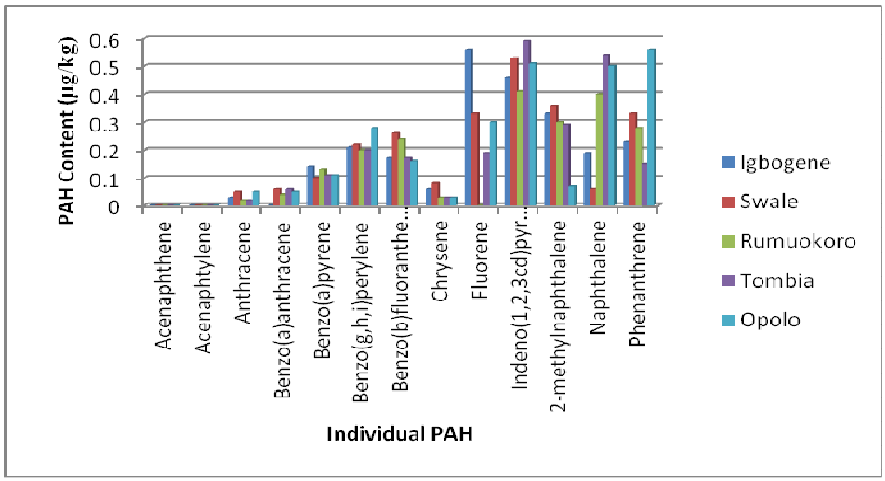

Fig. 2a: Individual PAH Content of Smoked Catfish (Clarias gariepinus) Samples

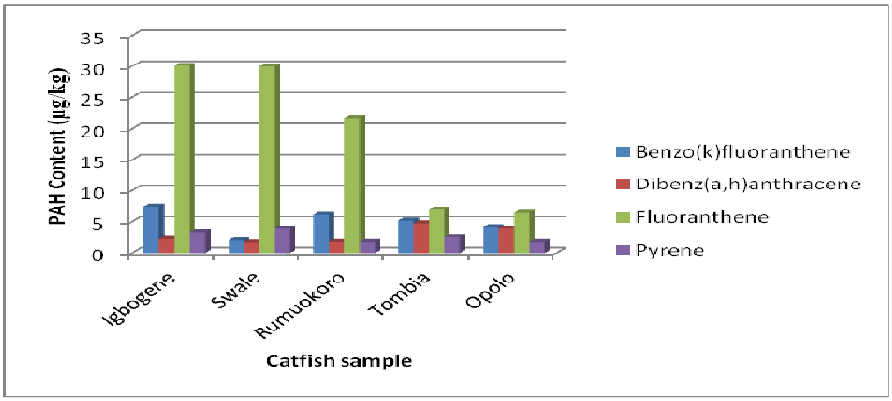

Fig. 2b: Individual PAH Content of Smoked Catfish (Clarias gariepinus) Samples

Statistical analysis using Student T Test and One Way analysis of Variance (SPSS 14.0 version) showed that there was significant difference between the PAH content of catfish samples from Opolo market and catfish samples from all the other markets with Opolo market samples having the lowest PAH content and samples from Igbogene recording the highest PAH content.

Discussion

This study has revealed the bacteria population, the types of bacteria and their frequency of occurrence, in smoked catfish (*Clarias gariepinus*) purchased from various markets in both Bayelsa and Rivers States of Nigeria. The highest and lowest heterotrophic bacterial load was recorded in fish

samples obtained from Igbogene and Tombia markets respectively, both in Bayelsa. The bacteria isolated and their frequency of occurrence were; *Bacillus* sp (34%), *Enterobacter* sp (7%), *Escherichia coli* (13%), *Micrococcus* sp (7%), *Pseudomonas* sp (13%), *Salmonella* sp (13%), and *Shigella* species (13%). On the other hand, only two oil degraders were isolated and they were *Bacillus* sp (71%) and *Pseudomonas* sp (29%). *E. coli* were isolated from fish samples from Igbogene, Rumuokoro and Opolo markets. *Enterobacter sp*. was found in fish samples from Igbogene and Opolo markets. On the other hand, *Salmonella* and *Shigella* species were isolated from smoked catfish samples from only Igbogene market. The occurrence of *Escherichia coli* in catfish from the aforementioned markets indicates feacal contamination which poses a serious health challenge to unsuspecting consumers. The occurrence of both feacal and non feacal coliforms in these fish may have been due to contamination of the fish products by handlers which portrays unhygienic handling, processing and distribution of catfish. This study complements that of Tiamiyu *et al*., (2011), who reported *Staphylococcus aureus*, *Bacillus sp*. *Salmonella sp*. and *Streptococcus* in the skin of *Clarias gariepinus*. Ibrahim *et al*., (2014) revealed that the following bacteria *Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermis, Salmonella epidermis, Salmonella typhii, Streptococcus* spp. and *Shigella* sp. were associated with smoked catfish (*Clarias gariepinus*). The presence of these organisms calls for caution because they are implicated in fish-borne human diseases (Babu, 2000). Bouriga *et al*., (2012) reported that mesophiles and total coliforms increase within smoking treatment with higher levels in the traditional smoking process. *Bacillus sp., Micrococcus sp*., and *Pseudomonas sp.* reported during this present study corroborates the report of Oku and Amakoromo (2013) who reported the presence of *Bacillus* and *Pseudomonas* sp. in smoked fish samples and Ibrahim *et al*., (2014) who reported *Micrococcus* species in both fresh and smoked fish.

The present study also revealed the proximate composition of the smoked catfish (*Clarias gariepinus*) purchased from the various markets. The highest and lowest fat content was recorded in catfish from Igbogene and Swale markets respectively; while the highest and lowest ash content was recorded in catfish from Swale and Igbogene markets respectively. On the other hand, the highest and lowest moisture content was recorded in catfish from Opolo and Tombia market respectively; while the highest and lowest crude protein content was recorded in catfish from Opolo and Swale market respectively. All the bacteria isolated in this study occurred in catfish from Igbogene market while all except *Salmonella* and *Shigella* occurred in Opolo market. While the highest and lowest heterotrophic bacterial load was recorded in fish samples obtained from Igbogene and Tombia markets respectively. The growth and proliferation of bacteria on the smoked catfish is therefore due to its moisture, crude protein and fat content (despite drying). The increase in

temperature during drying also favoured the growth and proliferation of bacteria. The organisms reported in this study are known to be heat resistant and thus were able to survive the process of heat treatment on the fish and also during storage. These observations were also reported by Ibrahim *et al*., (2014). The findings of this study is also in agreement with the findings of Abolagba and Iyeru (1998) who reported that lack of proper drying during smoking and lack of proper hygienic handling of smoked fish products usually result in very high microbial load.

Percentage ash content in *Clarias gariepinus* was highest in smoked fish obtained from Swale market and lowest in that obtained from Igbogene market. Salán *et al*., (2006) who worked on proximate analysis of both fresh and smoked *Clarias gariepinus* opined that the increase in ash content when fish are smoked is due to loss of humidity. This may account for the high bacteria count observed in fish from Igbogene due to the low ash content and high humidity. Doe and Olley (1983) reported that smoking resulted in concentration of nutrients like crude protein and fat.

This study has also revealed the hydrocarbon degrading bacteria and the polycyclic aromatic hydrocarbon (PAH) content in smoked catfish (*Clarias gariepinus*) purchased from various markets. Hydrocarbon utilizing bacteria isolated in the course of this study were *Bacillus* sp and *Pseudomonas* sp. This study shows that these species may be capable of degrading or utilizing PAHs due to their growth on mineral salt media containing crude oil as the sole carbon source.

The maximum total PAHs content of 45.57 μg/kg was obtained from fish sample from Igbogene while Opolo fish samples had the lowest total PAH content of 18.33 μg/kg. The varying total PAHs content may be attributed to the type of wood used and the smoking conditions such as the smoking temperature, and even the fat contents in the fish samples. This is similar to the findings of Kazerouni *et al*., (2001). Acenaphthene and acenaphthylene were absent in all the fish samples analysed. Other members of the PAHs group that have been classified as probable carcinogen by the Agency for Toxic Substances and Disease Control were present in the smoked fish samples in varying concentrations. The values of individual PAHs in some of the samples were higher than the European Standard for individual PAHs level (5 μg/kg) in foods. Flouranthene was available in all the examined fish samples with values ranging from 30.10 to 6.42 μg/kg. Benzo(a)pyrene was also available in all the examined fish samples with values ranging from 0.10 μg/kg in Swale catfish samples to 0.14 μg/kg in Igbogene catfish samples.

Values for Benzo(k)fluoranthene were 7.5 μ g/kg in Igbogene fish, 6.13 μ g/kg in Rumuokoro fish and 5.17 μ g/kg in Tombia fish. Flouranthene values (30.10 to 6.42 μ g/kg) were the highest recorded while anthracene values (0.02 to 0.05 μ g/kg) were the lowest recorded. The observed concentrations of PAHs detected in the catfish samples must have been imparted from the smoking process. Fish samples from Igbogene had the highest fat content (27.92%) and this was not unconnected with its PAHs value (45.47 μ g/kg) being the highest obtained during this study because of the lipophilic nature of PAHs.

Conclusion

The bacteria isolated from the catfish samples during this study were; *Bacillus* sp, *Enterobacter* sp, *Escherichia coli*, *Micrococcus* sp, *Pseudomonas* sp, *Salmonella* sp, and *Shigella* species. The oil degraders were *Bacillus* sp and *Pseudomonas* sp. The occurrence of both feacal and non feacal coliforms in these fish is attributed to contamination of the fish by handlers which portray unhygienic handling, processing and distribution of catfish. The occurrence of *E. coli* in catfish indicates feacal contamination which poses a serious health challenge to unsuspecting consumers. The presence of these organisms in the fish samples is worrisome since they are potential pathogens many of which have been implicated in food poisoning or fish-borne human diseases. This study shows that *Bacillus* and *Pseudomonas* species isolated from catfish exhibited growth on mineral salt media containing crude oil as the sole carbon source. They may be capable of degrading or utilizing PAHs and can therefore be useful in bioremediation processes. The observed presence of PAHs especially Benzo(a)Pyrene (which can be used as a marker for the occurrence and effect of these carcinogenic PAHs in food) in smoked catfish in Yenagoa and Port Harcourt metropolis calls for caution. Since this may pose health risk both to the handlers and the consumers because of the carcinogenic nature of PAHs and its ability to bioaccumulate in the body thereby predisposing the unsuspecting consumers to cancers. In view of the above challenges, other means of drying of fish is therefore advocated as to avert health hazards associated with wood fire smoking of fish.

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