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# Multiple In Vitro Bioassay Approach in Sediment Toxicity Evaluation: Masan Bay, Korea

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**Abstract** Extracts of 21 sediment samples from Masan Bay, Korea, used in an earlier chemical measurement, were screened for their ability to induce estrogen, – and dioxin – like gene expression using the E-Assay (+), DR-CALUX assay, respectively, and to inhibit acetylcholinesterase (AChE) activity using an in vitro *AChE* assay. Biological impact in the industry-rich inner bay is higher than outer bay. DDTs (0.65), coplanar PCBs (0.77), HCHs (0.64), PAHs (0.61) and APs (0.53) with good correlation to E-assay (+) are seen as environmental estrogens. The highest induction of DR-CALUX response was seen again at station M12 and 15 which received sewage effluents. PCDD/DFs gave the highest correlation (0.75). Interestingly, the M12 station at the sewage treatment outlet showed the highest activity. Among the targeted chemicals APs (0.66), PCBs (0.64), PAHs (0.61) and DDT (0.49) correlated well with the AChE bioassay. Spearman rank correlation on analytical and biochemical results affirmed the 'hot spots' and point sources (e.g., sewage treatment and industrial outfall) and suspected toxicants. Significant correlations between organo chlorine pesticides, PCBs, dioxins and alkylphenols and their biological effects were observed.

**Keywords** Sediment · Risk assessment · AChE · E-assay (+) · DR-CALUX

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Analytical measurement of priority pollutants in marine ecosystems is critical but not sufficient to predict their biological impact. Acute toxicity end-points such as survival and mortality of an organism do not always reveal the toxic potential, as most of the contaminants occur at low concentrations. Alternatively, in vitro bioassays may be used to determine the integrated toxic potency of a complex mixture of microcontaminants in the environment at relatively sensitive concentration range. In addition, such bioassays may detect mixture effects of compounds, even when the individual constituents of the mixture are present at concentrations too low to cause an effect or to be detected by chemical analysis (Hamers et al. 2010).

Masan Bay is situated in the southern part of Korea and is heavily polluted with various organic substances received from urban and agricultural runoff, industrial and domestic sewage discharges from nearby cities (Masan, Changwon and Jinhae), and wastes from shipping activity. This semi-enclosed bay is shallow with weak currents, allowing the pollutants to stagnate. Extensive chemical measurements have been carried out for chemicals such as PAHs, PCBs and dissolved sterols (Yim et al. 2005; Hong et al. 2010; Lee et al. 2011) but very limited effect-based biological testing has been carried out. An integrated ecotoxicological assessment was attempted in this work. The recombinant yeast assay (E-assay) is used in detecting xenoestrogens in sediment (Nishikawa et al. 1999). E-assay is constituted by cDNA of human $\alpha$-estrogen receptors (hER-$\alpha$) which bind to the xenoestrogen and induce the $\beta$-galactosidase (Hamblen et al. 2003). Dioxin-like activity was determined by the activation of arylhydrocarbon receptor (AhR) in a specific H4IIE-luc cell assay, the DR-CALUX cell bioassays are widely used in characterizing and evaluating Ah-R active compounds in sediment and other complex contaminant materials (Jung et al. 2008).

Inhibition of brain AChE activity has also been measured; AChE is inhibited by organophosphorus and carbamate pesticides, but recently it has been suggested as a biomarker for heavy metals and for petroleum exposure (Mora et al. 1999; Moreira et al. 2004; Moreira and Guilhermino 2005).

## Materials and Methods

Sediment samples were taken using a van-Veen grab, and surface sediments (~2 cm) were collected in glass bottles with Teflon lined caps. Immediately after collection, samples were frozen on dry ice, then transferred to the laboratory and stored at −20°C until analysis. The analytical procedure for organochlorines and polyaromatic hydrocarbons are described in Hong et al. (2003, 2009, 2010), Kannan et al. (2007, 2010) and Yim et al. (2005). The analytical procedure for butyltins is described in Shim et al. (1999) and Kim et al. (2011, 2002). To determine nonylphenol, nonylphenol monoethoxylate, and nonylphenol diethoxylate sediment samples were analyzed according to the methods of Li et al. (2008).

The recombinant yeast assay was conducted using the recombinant yeast strain *Saccharomyces cerivisae*, strain BJ3505 (hER 2ERE). Samples (10 μL) were transferred to 96-well plates. To dryness, chlorophenol red-b-D-galactopyranoside (CPRG) (200 μL) and yeast was added to each well. $\beta$-galactosidase activity was measured colorimetrically as it metabolized yellow CPRG intochlorophenol red. The phenol red was measured with a microplate reader as absorbency at 540 nm. In the study, estradiol-17$\beta$ was diluted in the range of $1–10^{-8}$ pg/mL for positive control. Ethanol alone (5 μL) was added as the "negative control" in incubation mixtures.

The assay procedure of DR-CALUX cell bioassays were conducted following the standard EPA methods 4435 (www.epa.gov/osw/hazard/testmethods). Sediment extracts were applied to monolayers of H1L6.1c3 cells. After the media removed, lysis buffer (30 μL) was added to each well then shook the plate for 1 min before inserting the plate into the microplate luminometer. For analysis of luciferase activity, 50 μL of luciferase substrate (luciferin) was added into each well following by quantitation of luciferase activity (light production) over a 15 s time period. 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD, dioxin) was diluted in the range of from $10^2$ pg/mL to $10^{-3}$ pg in positive control. Ethanol alone (5 μL) was used as the ''negative control" in incubation mixtures.

Brain acetylcholinesterase (AChE) bioassay of a benthic marine fish marbled sole (*Limanda yokohamae*) had been already characterized in terms of their kinetic properties (KM etc.) and optimal conditions (substrate concentration, protein concentration, pH etc.) to perform routine assays of AChE activity under pseudo-first order conditions (Jung et al. 2007). AChE activity was expressed as activity of the PMS towards the diagnostic substrate acetylthiocholine in the Ellman reaction (Ellman et al. 1961). Enzyme preparations in buffer were usually pre-incubated with extract for 30 min., after which 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and substrate were added. A microplate reader method was developed based on absorbance measurements using a filter with a transmission maximum at 415 nm, this was calibrated against spectrophotometer readings at 412 nm (the absorbance maximum of the reaction product 5-thio-2-nitrobenzoic acid) using thiocholine positive controls. Ethanol alone (5 μL) was added as the ''negative control" in incubation mixtures. At least three replicates of each assay were performed. ΣCongeners/isomers or individual chemicals determined in sediments of 21 stations were correlated with bioassay results using Spearman's correlation with IBM SPSS Statistics (v16.0, Chicago, IL, USA).

## Results and Discussion

The average concentrations (ng $g^{-1}$ dry weight) of target chemicals were in the following order: APs – 12,000 ± 430; PAHs – 6,500 ± 140; BTs – 2,500 ± 100; PCBs – 150 ± 6.5; Coplanar PCBs – 0.78 ± 0.05; chlordanes – 60 ± 3.4; PCDFs – 30 ± 3.6; PCDDs – 20 ± 1.4; endrin – 5.0 ± 0.26; PBDEs – 4.0 ± 0.29; HCHs – 2.24 ± 0.22; DDTs – 0.92 ± 0.06; aldrin – 0.90 ± 0.06; dieldrin – 0.90 ± 0.06; endosulfan II – 0.79 ± 0.06; mirex – 0.27 ± 0.02; HCB – 03 ± 0.01 (Table 1). These extracts were tested for their biological potency.

### E-Assay (+)

Among the 21 stations sampled for biological activity, the following 10 stations showed estrogenicity in that order: M12> M15> M5> M8> M9> M6> M10> M20> M18> M21 (Fig. 1a). Most interestingly, station 12 which received effluents from a sewage treatment plant that treats 260,000 ton $d^{-1}$ of domestic (90 %) and industrial (10 %) wastes day showed the highest biological activity. Station M15 receives sewage outfall to a lesser degree (60,000 ton $d^{-1}$). Biological impact in the industry-rich inner bay is higher than outer bay. Among the15 targeted contaminants in Masan Bay, 10 chemical species showed good correlation with estrogenic potency (Table 2). Chemicals such as DDTs (0.65), coplanar PCBs (0.77), HCHs (0.64), PAHs (0.61) and APs (0.53) with good correlation to E-assay (+) are seen as environmental

**Table 1** Mean concentration of target chemicals (ng $g^{-1}$ dry weight) in Masan Bay sediments

| Stations | APs | PAHs | BTs | PCBs | Co-PCBs | CHLS | PCDFs | PCDDs | Endrin | PBDEs | HCHs | DDTs | Aldrin | Dieldrin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 934 | 304 | 318 | 9.62 | 0.027 | 2.17 | 0.69 | 0.82 | 0.3 | 0.041 | 0.049 | 0.045 | 0.045 | 0.04 |
| 2 | 892 | 378 | 329 | 10.1 | 0.069 | 2.69 | 037 | 0.33 | 0.3 | 0.805 | 0.015 | 0.04 | 0.04 | 0.04 |
| 3 | 645 | 577 | 305 | 20.6 | 0.045 | 8.54 | 0.82 | 1.11 | 0.33 | 0.038 | 1.01 | 0.15 | 0.13 | 0.13 |
| 4 | 409 | 232 | 125 | 4.75 | 0.028 | 1.41 | 0.4 | 0.57 | 0.1 | 0.031 | 0 | 0.031 | 0.031 | 0.03 |
| 5 | 632 | 707 | 73 | 23.8 | 0.194 | 14 | 0.68 | 1.37 | 0.38 | 0.077 | 0.17 | 0.21 | 021 | 0.21 |
| 6 | 643 | 292 | 175 | 7.19 | 0.032 | 2.33 | 0.5 | 0.59 | 0.14 | 0.114 | 0.023 | 0.052 | 0.052 | 0.05 |
| 7 | 440 | 518 | 152 | 6.01 | 0.019 | 1.48 | 0.19 | 0.23 | 0.15 | 0.035 | 0 | 0.026 | 0.026 | 0.03 |
| 8 | 527 | 315 | 79 | 13.1 | 0.034 | 2.37 | 0.45 | 0.72 | 0.19 | 0.084 | 0.089 | 0.043 | 0.043 | 0.04 |
| 9 | 317 | 295 | 50 | 3.21 | 0.027 | 1.9 | 0.29 | 0.91 | 0.48 | 0.067 | 0.12 | 0.034 | 0.034 | 0.03 |
| 10 | 666 | 340 | 91 | 7.09 | 0.032 | 2.08 | 0.86 | 0.89 | 0 | 0.139 | 0.12 | 0.082 | 0.082 | 0.08 |
| 11 | 801 | 238 | 149 | 13.55 | 0.033 | 2.53 | 3.1 | 1.51 | 0 | 0.104 | 0.026 | 0.09 | 0.09 | 0.09 |
| 12 | 2191 | 386 | 257 | 9.48 | 0.057 | 3.53 | 16.73 | 6.89 | 0 | 0.245 | 0.18 | 0.009 | n.d. | n.d. |
| 13 | 490 | 214 | 53 | 2.41 | 0.01 | 0.85 | 0.97 | 0.63 | 0.46 | 0.018 | 0 | 0 | n.d. | n.d. |
| 14 | 395 | 224 | 37 | 1.32 | 0.007 | 0.77 | 0.92 | 0.77 | 0.45 | 0.022 | 0 | 0 | n.d. | n.d. |
| 15 | 381 | 351 | 162 | 5.47 | 0.119 | 7.79 | 0.26 | 0.49 | 1.01 | 0.668 | 0.19 | 0.11 | 0.11 | 0.11 |
| 16 | 319 | 193 | 60 | 6.1 | 0.02 | 1.28 | 0.17 | 0.52 | 0.43 | 0.035 | 0.081 | 0 | n.d. | n.d. |
| 17 | 483 | 196 | 39 | 1.65 | 0.01 | 1.17 | 0.12 | 0.33 | 0 | 0.017 | 0.024 | 0 | n.d. | n.d. |
| 18 | 288 | 171 | 25 | 0.67 | 0.006 | 0.48 | 0.24 | 0.5 | 0 | 0.011 | 0.047 | 0 | n.d. | n.d. |
| 19 | 265 | 202 | 22 | 0.72 | 0 | 0.59 | 0.2 | 0.47 | 0 | 0.001 | 0.072 | 0 | n.d. | n.d. |
| 20 | 192 | 187 | 18 | 1.14 | 0.006 | 0.63 | 0.17 | 0.51 | 0 | 0.025 | 0.025 | 0 | n.d. | n.d. |
| 21 | 142 | 176 | 11 | 0.026 | 0 | 0.036 | 0.16 | 0.5 | 0 | 1.06 | 0 | 0 | n.d. | n.d. |
| Sum | 12051 | 6494 | 2530 | 148 | 0.78 | 59 | 28 | 21 | 4.72 | 3.64 | 2.24 | 0.92 | 0.9 | 0.9 |
| STDev | 430 | 142 | 104 | 6.48 | 0.05 | 3.36 | 3.58 | 1.39 | 0.26 | 0.29 | 0.22 | 0.06 | 0.06 | 0.06 |

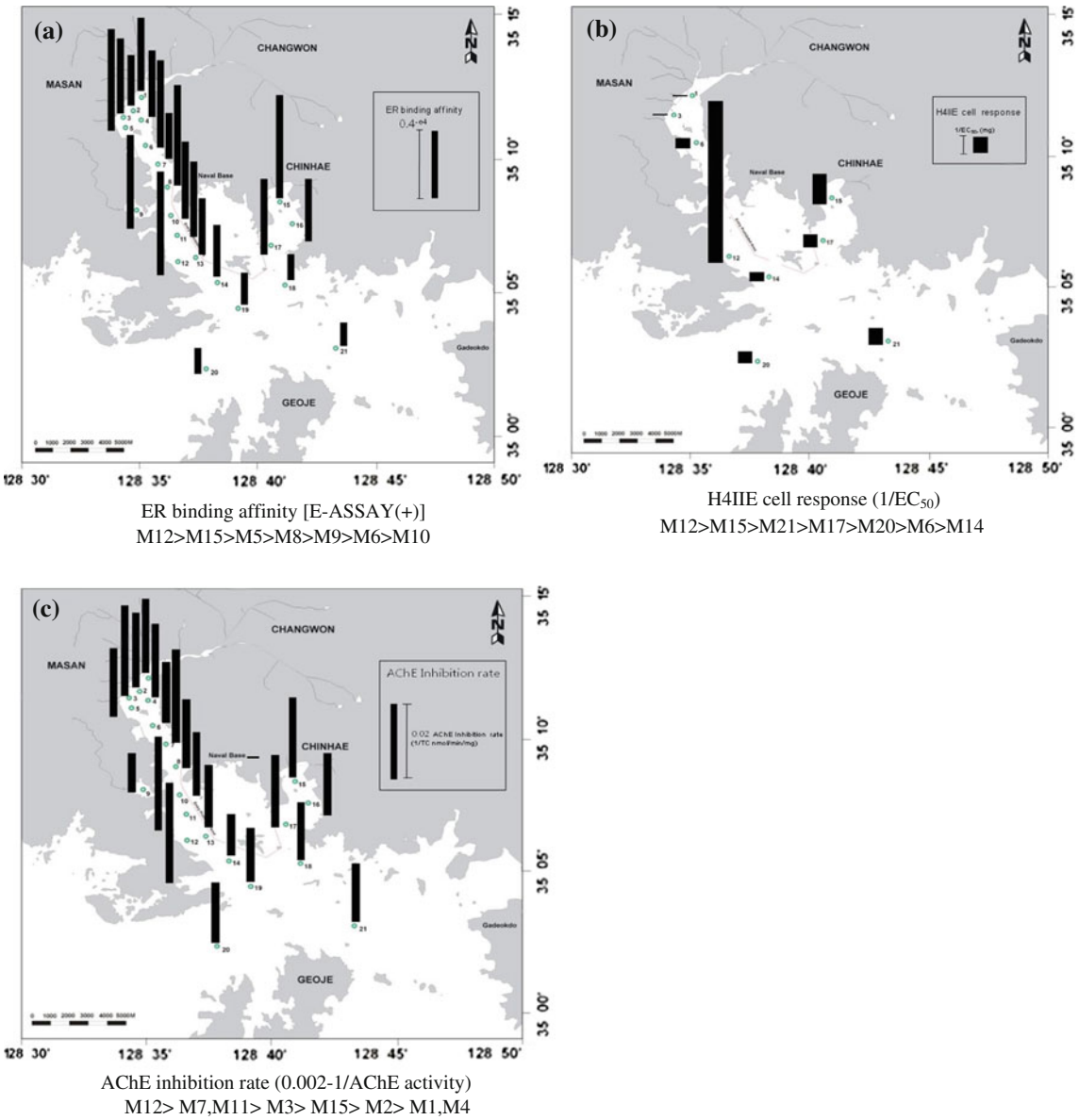
*n.d.* Non-detectable

*APs* Alkylphenols, *PAHs* Polyaromatic hydrocarbons, *BTs* Butyltins, *PCBs* polychlorinated biphenyls, *Co-PCBs* coplanar PCBs, *CHLs* Chlordanes, *PCDFs* polychlorinated dibenzofurans, *PCDDs* polychlorinated dibenzo-p-dioxins, *PBDEs* polybrominated diphenyl ethers, *HCHs* hexachlorocyclohexanes, *DDTs* Dichlorodiphenyltrichloroethanes

estrogens. For example, three PAHs, chrysene (CH), benz[a]anthracene (BaA), and benzo[a]pyrene (BaP), have been shown to elicit estrogen like responses in an in vitro gene expression assay (Clemons et al. 1998). p,p′-DDT has been reported to cause weak estrogen-like responses in vitro (Soto et al. 1994). However, such responses were reported at concentrations generally exceeding 1 μg $g^{-1}$ (Soto et al. 1994). The concentrations of target OC pesticides (Table 1) in Masan Bay sediment extracts were less than 60 ng $g^{-1}$. Known environmental estrogen mimics like nonylphenol showed a correlation of 0.53. Instead, chlordanes which are not estrogenic (Wandji et al. 1998) showed relatively good correlation (0.77). PCBs have both estrogenic and anti-estrogenic properties, especially coplanar PCBs that showed a correlation of 0.77 are in fact estrogenic properties (Vakharia and Gierthy 1999). PAHs are suspected to exhibit both estrogenic and anti-estrogenic properties (Gozgit et al. 2004) (Table 2). This is possible only if we presume that enzyme activity is influenced by both target and non-target chemicals (that escape instrumental detection) in a complex interactive manner: synergistic, antagonistic, inhibitive and additive. This needs further study in the future.

## DR-CALUX bioassay

PCDDs/DFs and PCBs are known AhR agonists (Safe 1990). Among the 21 sediment samples tested, 9 samples gave positive response in this assay in the following order: M12> M15> M21> M17> M20> M6> M14> M3> M1 (Fig. 1b). The highest induction was seen again at station M12 and 15 which received sewage effluents. PCDD/DFs gave the highest correlation (0.75). The possible impact of sewage treatment effluent and inner bay industrial/domestic waste runoff was predicted before (Kannan et al. 2007, 2010; Hong et al. 2009). Biological response in rockfish at Masan Bay confirmed the potential impact by comparing the different cage sites (Masan bay and Haegeumgang), hepatic CYP1A and EROD activity in fish were significantly higher in Masan Bay after the caging experiment (Jung et al. 2008). Present results corroborate well with the previously predicted and the observation.




ER binding affinity [E-ASSAY(+)]
M12>M15>M5>M8>M9>M6>M10

H4IIE cell response ($1/EC_{50}$)
M12>M15>M21>M17>M20>M6>M14

AChE inhibition rate (0.002-1/AChE activity)
M12> M7,M11> M3> M15> M2> M1,M4

**Fig. 1** Spatial distribution biological activity in sediments from Masan Bay, Korea as revealed by in vitro bioassays. **a** Estrogen receptor binding affinity, **b** H4IIE cell response ($1/EC_{50}$) and **c** AChE activity inhibition rate (1/AChE activity)

## AChE Assay

The inhibition of acetyl cholinesterase (AChE) activity has been utilized recently as a tool for marine ecotoxicology (Jung et al. 2007, 2008). Among the 21 sediment samples tested 9 of them showed positive response to AChE bioassay in the following order: M12> M7, M11> M3> M15> M2> M1, M4> M17> M8 (Fig. 1c). Once again, the M12 station at the sewage treatment outlet showed the highest activity. ChEs are inhibited by organophosphorus and carbamate pesticides (e.g., Giacobini 2000) and the area receives some agricultural runoff from its hinterland. In addition, ChE activities in fish may be affected by hitherto unidentified compounds in industrial wastes (Payne et al. 1996). For example, AChE activity has been shown to decrease in fish exposed to herbicides containing glyphosate (Glusczak et al. 2006; Sancho et al. 2000; Menéndez-Helman et al. 2012). Among the targeted chemicals APs (0.66), PCBs (0.64), PAHs (0.61) and DDT (0.49) correlated well with the AChE bioassay (Table 2). Alkylphenolic compounds suppressed AChE activity in rat neuronal cell line PC12 and in guppies (Talorete et al. 2001; Li 2008). Although it is known that PCBs are not ChE inhibitors, negative correlations were obtained between PCB content and liver total ChE activity in bream (*Abramis brama*) (Chuiko et al. 2007). It has been well

established that AChE plays an integral role in cholinergic nerve transmission and is the target site of inhibition by organophosphorous and carbamate insecticides. It is known that AChE is not a target enzyme for organochlorinated insecticides. In spite of this fact, Spearman analysis showed a correlation of 0.49 between AChE activity and DDTs. However, if modulation of these enzymes is controlled by other chemicals and the synergistic action of mixed pesticides occurs, the correlation may be justified (Bocquené et al. 1995).

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**Table 2** Correlation values (r-values) for the response of bioassays and chemical concentration in Masan Bay sediments

| Bioassay | Chemical | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HCHs | CHLs | DDTs | Dieldrins | Endrin | Endosulfan II | Mirex | PCBs | PCBs (arochlor) | Co-PCBs | PAHs | BTs | PBDEs | PCDDs | PCDFs | APS |
| RE-H4IIE | | | | | | | | | | | | | | | | |
| r-value | 0.07 | 0.26 | 0 | 0.20 | 0.08 | 0 | 0 | 0.23 | 0.23 | 0.36 | 0.50 | 0.16 | 0.23 | 0.74 | 0.79 | 0.63 |
| p-value | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | 0.02 | $p > 0.05$ | $p > 0.05$ | 0.01 | 0.01 | 0.01 |
| AChE | | | | | | | | | | | | | | | | |
| r-value | 0.2 | 0.67 | 0.49 | 0 | 0 | 0.05 | 0.43 | 0.64 | 0.65 | 0.64 | 0.61 | 0.75 | 0.00 | 0.13 | 0.55 | 0.66 |
| p-value | $p > 0.05$ | 0.01 | 0.02 | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | 0.02 | 0.02 | 0.02 | 0.03 | 0.01 | $p > 0.05$ | $p > 0.05$ | 0.01 | 0.01 |
| E-ASSAY (+) | | | | | | | | | | | | | | | | |
| r-value | 0.64 | 0.77 | 0.65 | 0.14 | 0.28 | 0.01 | 0.22 | 0.62 | 0.63 | 0.77 | 0.61 | 0.48 | 0.01 | 0.55 | 0.45 | 0.53 |
| p-value | 0.02 | 0.00 | 0.01 | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | $p > 0.05$ | 0.03 | 0.03 | 0.01 | 0.03 | 0.02 | $p > 0.05$ | 0.03 | 0.04 | 0.01 |

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