Estrogens are ancient molecules that act as hormones in vertebrates and are biologically active in diverse animal phyla. Sewage contains natural and synthetic estrogens that are detectable in streams, rivers, and lakes. There are no studies reporting the distribution of steroidal estrogens in marine environments. We measured estrogens in sewage, injection-well water, and coastal tropical and offshore tropical water in the Pacific Ocean, western Atlantic Ocean, and Caribbean Sea. Concentrations of unconjugated estrone ranged from undetectable (<40 pg/L) in the open ocean to nearly 2,000 pg/L in Key West, Florida, and Rehoboth Bay, Delaware (USA); estrone concentrations were highest near sources of sewage. Enzymatic hydrolysis of steroid conjugates in seawater samples indicated that polar conjugates comprise one-half to two-thirds of “total estrone” (unconjugated plus conjugated) in Hawaiian coastal samples. Adsorption to basalt gravel and carbonate sand was less than 20% per week and indicates that estrogens can easily leach into the marine environment from septic fields and high-estrogen groundwater. Of 20 sites (n = 129 samples), the mean values from 12 sites were above the threshold concentration for uptake into coral, indicating that there is a net uptake of anthropogenic steroidal estrogens into these environments, with unknown impacts. Key words: effluent, environmental, estrogen, estrone, marine, radioimmunoassay, sewage. Environ Health Perspect 111:531–535 (2003). doi:10.1289/ehp.5233 Available via http://dx.doi.org [Online 31 October 2002]
ranged between 0 and 35.5%, with most samples in the range of 20–35.5%, representing distances of tens to hundreds of meters offshore. Not all samples were measured for salinity and silicate; therefore, we used distance, salinity, and silicate to rank each sample 1 to 5 according to its proximity to sewage effluent (1 = open ocean, 5 = sewage effluent; Table 1). Sewage treatment facility samples (200 mL) were collected from the Lahaina–Napili Wastewater Reclamation Facility (n = 4); sewage samples included raw influent, postsecondary clarifier, R-1 irrigation water (R-1 is a rating for the highest treatment level of wastewater that can be reused), and injection-well effluent. Samples were collected July 1998 through June 1999 (Table 1). All samples were filtered through glass microfiber filters (GF/C) and stored at −20°C until analysis for estrone.

Extraction and assay. Estrogens were concentrated by chromatography and assayed using a highly specific radioimmunoassay for estrone, which has been validated previously for a variety of vertebrate plasma and tissues and coral tissue (26–28). Sep-Pak C18 reverse-phase chromatographic columns (3 cc; Waters Corp., Milford, MA) were arranged on a vacuum manifold attached to a vacuum pump and were then conditioned with 5 mL methanol and 5 mL water (both HPLC grade). Seawater (250 mL) and sewage (10 mL) samples were chromatographed. Estrogenes were eluted from the columns with 3 mL diethyl ether; the ether extract was flash-frozen, decanted, and concentrated to dryness with prepurified nitrogen. To calculate the extraction efficiency, 100 µL of titrated estrone was added to 250 mL aliquots of seawater, which were vortexed and chromatographed. Mean extraction recovery was 88 ± 3%. The radioimmunoassay antiserum was developed in sheep against estrone-3-carboxymethylloxime–gelatin (29). Cross-reactivities of the antiserum were measured for nine C18 compounds, nine C19 compounds, and seven C21 compounds. All C19 and C21 compounds yielded < 0.1% cross-reactivities; estrone sulfate and estrone were 100% immunoreactive. Estrone-3-glucosiduronate was 51%, whereas all forms of estradiol and estriol were < 0.1% immunoreactive (29). Ethinyl estradiol was cross-reactive at 0.1%. Dried extracts were incubated for 1 hr at 37°C with 100 µL of tritiated tracer ([H]-1,2,6,7-estrone: specific activity, 53.5 Ci/mmol) and 100 µL of diluted antiserum. Bound estrogens were separated from free estrogens at the end of the incubation using 1 mL of 0.4% Norit-A charcoal in phosphate-gelatin buffer. After 10 min the mixture was centrifuged for 10 min at 2,500 rpm at 4°C. An aliquot of the supernatant (500 µL) was added to a scintillation cocktail, and each tube was counted for 5 min. Estrone concentrations were read off a standard curve that had a log-logit transformation applied to it (30).

Nonspecific binding of the assay was 5.1 ± 0.7%, and the lower detection limit for seawater samples was 40 pg/L. We further validated the estrone assay for sewage in seawater by showing standard additions of raw sewage, ether-extracted sewage, and filtered seawater were parallel with a standard curve of estrone concentration. To determine parallelism with the standard curve, twenty 1-mL aliquots of unfiltered sewage were chromatographed and assayed as described above. The estrone-containing extracts were added to tubes containing dried standards, and the ether was evaporated. For filtered sewage, 100 µL aliquots were added directly to tubes containing dried standards and assayed as usual. For the filtered seawater, 250 mL was chromatographed and extracted separately, then added to a dried standard curve.

To determine the effect of GF/C filtration on estrone concentration, five filtered and five unfiltered sewage effluent samples (1 mL) were chromatographed and assayed for estrone. Estrone was not significantly different between filtered (9,800 ± 2,000 pg/L) and unfiltered (8,300 ± 5,600 pg/L) sewage. Twenty-four filters from a variety of sewage (10 mL) and seawater (250 mL) samples were assayed to determine whether particles carry estrogen. None of the filters had detectable estrone. We conclude that estrone is in the dissolved fraction of water and that filtering does not interfere with estrogen detection. Additionally, estrone amount showed a strong linear relationship to sample volume (0.5–10 mL, n = 7; r2 = 0.98, p < 0.001), indicating that estrone concentrations were not a function of sample volume.

Sulfate experiments. To determine the relative concentrations of conjugated and unconjugated forms of estrone, water samples from three different sources around Oahu, Hawaii (Kaneohe Bay ~500 m offshore, Coconut Island lagoon, and sewage effluent) were selected to represent a wide range of estrogen concentrations and proximity to sewage effluent. Each sample was assayed in triplicate for both total (unconjugated and polar conjugates) and unconjugated estrone. Sample volumes were 250 mL (Kaneohe Bay and Coconut Island lagoon) and 30 mL (sewage effluent). Helix pomatia extract (type H-2; sulfatase activity, 2,000–5,000 U/mL; β-glucuronidase activity, ~100,000 Sigma units/mL at pH 5.0) was obtained from Sigma (St. Louis, MO). For analysis of total estrone, one set of samples was buffered with 0.1 M sodium acetate and acidified with 20% acetic acid to pH 5.0. Diluted H. pomatia extract (1 mL of a 1% crude solution in 0.2 M sodium acetate, pH 5.0) was added to each of these samples. To measure total estrone, samples were first incubated at 37°C overnight with continuous shaking. The next day, these samples, and a second parallel set of samples (to measure unconjugated estrone), were chromatographed, extracted, and assayed for estrone, as described above.

Basal carbonate experiments. The ability of basals and carbonates (the main geologic substrates of Pacific islands) to adsorb estrone was investigated by incubating sewage influent (200 mL) in polyethylene bottles containing basalt (60 g; Niu black cinders, Aloha Agricultural Consultants, Honolulu, HI) or carbonate (60 g, coarse-grained sand collected

Table 1. Description of samples analyzed for estrone concentration.

| PSE | n | Date of collection | Site: description |
|-----|---|--------------------|------------------|
| 1   | 7 | 98–99              | Open ocean: Florida, Hawaii, French Polynesia, Marianas Islands |
| 2   | 1 | 7/98               | Biosphere 2 Ocean, Arizona: Enclosed, 2,650 m² reef mesocosm |
| 2   | 6 | 2/99               | Molokai, Hawaii: fishing coral reef, uninhabited and land |
| 2   | 4/99|                  | Rangiroa, French Polynesia: large atoll lagoon, inhabited motus |
| 2   | 5/99|                  | Northwestern Hawaiian Islands: wildlife refuge with fishing reefs, field station |
| 2   | 6 | 0/98               | South Big Pine Key, Florida: offshore patch reef and seagrass meadow |
| 2   | 6 | 1/99               | Key Largo, Florida: offshore reef |
| 2   | 5 | 3/99               | Tinian, Marianas Islands: fishing reef near town |
| 3   | 7 | 11/98, 1/99        | West Maui, Hawaii: rocky shoreline with macroalgae mixed with sand beaches; some cesspools |
| 3   | 5 | 3/99               | Kaneohe Bay, Hawaii: coral reef lagoon adjacent to city |
| 3   | 1 | 3/99               | Coconut Island, Hawaii: lagoon within Kaneohe Bay but adjacent to marine lab |
| 3   | 4 | 6/99               | Key West Channel, Florida: channel with hard bottom near major harbor |
| 3   | 4/99|                  | Moorea, French Polynesia: inshore reef near large resort |
| 4   | 2 | 3/99               | Guam, Marianas Islands: Tumon Bay, coral reef lagoon near resort complex |
| 4   | 4 | 2/99               | Maalaea Bay, Maui, Hawaii: harbor and bay with sandy bottom, condominiums with shallow injection wells |
| 5   | 2 | 6/99               | Key West Harbor, Florida: harbor with mud bottom, adjacent to city |
| 5   | 1 | 1/99               | Golf course, Maui, Hawaii: pond in center of course, R-1 irrigation water |
| 5   | 3 | 3/99               | Rehoboth Bay, Delaware: estuary bay with sewage diffuser outfall |
| 5   | 4 | 1/99               | Sewage treatment facility, Maui, Hawaii: influent, secondary clarifier, R-1 and injection well effluent |

Abbreviations: n, number of samples collected; PSE, proximity to sewage effluent—ranked from 1 (open ocean) to 5 (sewage effluent).
from the Coconut Island beach, sun-bleached) or in empty control bottles, each treatment in triplicate. Aliquots (10 mL) were withdrawn on days 1, 2, 4, and 8 and assayed for estrone concentration. In a second experiment, sewage influent (50 mL) was added to polyethylene bottles containing either basalt (15 g) or carbonate (15 g) or to empty control bottles, each treatment in triplicate. Upon addition, the mixtures were incubated at 20°C, after which they were assayed for estrone concentration. In a second experiment, sewage influent (50 mL) was added to polyethylene bottles containing either basalt (15 g) or carbonate (15 g) or to empty control bottles, each treatment in triplicate. Upon addition, the mixtures were incubated at 20°C, after which they were assayed for estrone concentration.

**Results**

**Sulfatase experiments.** Preliminary experiments showed that although our antibody would bind to estrone sulfate, estrone sulfate was not collected during our extraction procedure (without hydrolysis). In contrast, when samples were spiked with estrone sulfate and subjected to hydrolysis with H. pylori extract, the recovery of estrone sulfate was >90%. In the three sample types analyzed (oceanic seawater, Coconut Island lagoon seawater, and sewage effluent), the unconjugated estrone was 34–54% of the total estrone measured after hydrolysis (Table 2). Interestingly, the sewage sample had a higher proportion of unconjugated estrogens (54%) than the two seawater samples (34–35%).

**Basal carbonate experiments.** The concentration of estrone in sewage was not significantly different from day 1 to day 8, indicating relatively little adsorption onto inorganic mineral surfaces. In the experiment with dissolved tritiated estrone, the radioactive counts in carbonate treatments were lower, but not significantly (9%), than control, and in basalt treatments they were significantly lower (18%) than control (Figure 1), indicating that a small fraction of estrogen in raw sewage can adsorb to coarse sands in an aquifer. Results of the unlabeled incubations were no different from those of the radiolabeled incubations, indicating that metabolic degradation or alteration did not significantly affect the behavior of estrone around different substrates.

**Field sampling.** Estrone concentration in raw sewage influent to a sewage treatment plant (West Maui, HI) was 77,000 ± 14,000 pg/L. After processing through the secondary clarifier, a single sample had a concentration of 19,000 pg/L. Sewage effluent pumped from the sewage treatment plant into local injection wells was 3,000 ± 400 pg/L, whereas R-1 water, primarily used for irrigation of local golf courses, was 7,700 ± 700 pg/L. Therefore, estrone concentration of groundwater leaching into coastal oceans can be expected to have an initial concentration of several nanograms per liter and to dilute to open ocean concentrations 500-fold lower (Figure 2).

Sampling sites in embayments with sewage sources had one to two orders of magnitude higher estrone concentrations than did open ocean water (Table 1). Open-ocean water samples from tropical regions near the Hawaiian Islands, Marinas Islands (Guam and Tinian), French Polynesia (Rangiroa and Moorea), and Florida Keys averaged 52 ± 15 pg/L and were the lowest estrone concentrations in this study, some of which were nondetectable in our assay. Interestingly, the Biosphere 2 ocean, a large (2,650 m³, 710 m³), completely contained and isolated coral reef mesocosm inside Biosphere 2 (Tucson, AZ), had the second lowest estrone concentration of 66 pg/L, indicating that the high residence time of water (8 years) over this particular coral reef community does not necessarily create high concentrations of estrogen. Rangiroa Lagoon, a very large atoll lagoon in French Polynesia, had a mean concentration of 400 ± 18 pg/L. Water samples from the dry, south coast of Molokai, Hawaii, had estrone concentrations only 2-fold higher than those of the open-ocean samples (52 vs. 120 ± 18 pg/L). The mean of 70 samples from the west coast of Maui, which is exposed to the open ocean but has sewage outfalls and agricultural runoff, averaged 160 ± 10 pg/L. Water samples from two sites in the Florida Keys—Key Largo and South Big Pine Key—had estrone concentrations of 260 ± 51 pg/L, 5-fold above oceanic values. Water samples collected near beaches with septic fields and public toilet facilities on remote islands of Tinian, Marianas Islands, and Tern Island, northwestern Hawaiian Islands, had estrone concentrations 6-fold higher than open-ocean samples (310 ± 28 pg/L for Tinian samples and 350 ± 91 pg/L for Hawaiian samples). Similarly, estrone concentrations in samples collected within 50 m of the beach resorts of Tumon Bay, Guam, and a large resort on Moorea were 10-fold above those of ocean samples (Guam, 480 and 710 pg/L; Moorea, 610 pg/L). Even the small lagoon of Hawaii Institute of Marine Biology on Coconut Island

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**Table 2. Estrone concentration (pg/L) in seawater samples collected from Kaneohe Bay (ocean), Coconut Island lagoon (shore), Hawaii, and sewage effluent (sewage).**

| Source      | Total estrone | Unconjugated estrone |
|-------------|---------------|-----------------------|
| Ocean       | 500 ± 140     | 110 ± 96              |
| Shore       | 1,680 ± 210   | 590 ± 45              |
| Sewage      | 20,050 ± 2,200| 11,000 ± 3,600        |

*Values are mean concentration ± SE. Total estrone was measured by radiomununassay following enzymatic hydrolysis and extraction. Unconjugated estrone was measured without hydrolysis. Conjugated estrone can be calculated as the difference between the total estrone and the unconjugated estrone.

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**Figure 1.** Radioactive estrone counts (cpm ± SE) throughout 8 days in three treatments: empty bottles as a control and bottles containing basalt or carbonate sands.

**Figure 2.** Mean (± SE) estrone concentrations for each site presented in order of increasing proximity to sewage effluent. The lower limit of detection for estrone is 40 pg/L. See Table 1 for description of sites.
in Kaneohe Bay, Oahu, had concentrations 10-fold above those of ocean water (580 pg/L) and nearly 3-fold above those in Kaneohe Bay water (210 ± 31 pg/L).

Embayments and lagoons with known sources of sewage from septic fields and injection wells had levels that were within a factor of 10 of sewage effluent: Key Largo shore (850 pg/L); Maalaea Bay, Maui (690 ± 126 pg/L); Big Pine Key Canal (660 ± 254 pg/L); Key West Channel (810 ± 85 pg/L); and a golf course pond using R-1 irrigation water on Maui (830 pg/L). The highest values of estrogen in this study were 30-fold above those of ocean values, and both were from shallow embayments with known sewage inputs: Rehoboth Bay (1,870 ± 247 pg/L), and Key West Harbor (1,580 ± 189 pg/L).

**Discussion**

Enzymatic hydrolysis of conjugated estrogen in seawater samples demonstrated that approximately one-half to two-thirds of total estrogen in the samples occurs as polar conjugates. Environmentally relevant concentrations of dissolved estrogen can be removed from the water column by reef-building corals (2), but it is not yet known whether estrogen conjugates can be removed in a similar manner by corals or other organisms. Polar conjugates of estrogens are common excretory products, and they are relatively inactive in vertebrates compared with unconjugated parent compounds. Polar estrogen conjugates do not show high affinity for nuclear estrogen receptors (3,4); however, various aerobic and anaerobic bacteria can hydrolyze these esters under appropriate conditions (3,2) and could provide a continual source of unconjugated estrogens. Although these pathways have been demonstrated experimentally, additional research is needed to determine the rates of these processes in marine ecosystems.

Between 9 and 18% of the estrone added to samples adsorbed to the surface of coarse carbonate sand or basalt gravel over the course of a week. This result is consistent with our previous finding that estrone (1,000–2,000 pg/L) dissolved in seawater and exposed to full sunlight did not significantly degrade over the course of 1 week, and adsorbed only slowly to the sides of a tank or dead coral skeletons (3,2). More estrone likely would have adsorbed to both mineral surfaces if the surfaces were conditioned with a “biofilm” of bacteria and microalgae.

We have previously used estrone as a tracer of nitrogen from septic fields into coastal waters off Maui (29). Estrogens released into coastal environments with sewage are one component of a diverse mixture of compounds, many of which degrade rapidly, rendering them poor indicators of sewage in the marine environment. Estrone increased with proximity to sewage effluent, was low (non-detectable in some cases) in open-ocean seawater, and appears to be relatively stable in the marine environment, making it a useful indicator of the presence of sewage effluent.

Estrogens in the coastal marine environment possibly affect reproductive biology, through blocked embryonic development (6), altered enzymatic activities (7,8), or cellular damage or apoptosis (10,11). Additional work is needed to describe the distribution and in situ concentrations of these estrogenic compounds, including the relative abundance of various steroidal components. Estrogens in the picomolar range of concentrations can alter the development of aquatic organisms (3,33,34). Considering that many invertibrates are at the base of aquatic food chains, human-derived estrogens in marine ecosystems could greatly affect ecosystem function. Detailed sampling will be required to establish fluxes of estrogen, possible uptake and accumulation, and physiologic and egestion responses of marine organisms; nevertheless, these data clearly indicate that many marine coastal environments could have large pools of these environmentally persistent molecules.

Experiments on uptake of estrone into communities of corals revealed that estrone is removed from water in proportion to its concentration, and the proportionality constant is close to the theoretical physical maximum (2). When estrogen concentrations in seawater drop to approximately 300 pg/L, the rate of estrone uptake is balanced by release. Thus, in general, concentrations of greater than 300 pg/L estrone will result in net uptake and possible accumulation into the reef benthos. Twelve of the 20 sites sampled in the present study had a mean value above 300 pg/L, indicating that many sites may be affected by elevated estrogens in nearshore waters.

In considering possible effects of estrogens or estrogen mimics on wildlife, it is not obvious which estrogenic compound is of greatest potential concern and should be monitored. Although estradiol is the most biologically active natural steroidal estrogen in mammals, estrogen may be present at a higher concentration and easier to detect analytically. In addition, diverse animals and microorganisms can interconvert estrone and estradiol (32,35,36). An argument can also be made for the determination of estrogenic activity using bioassays such as proliferation of breast cancer cells or production of vitellogenin (22,37,38).

Although we recognize the validity of these approaches, breast cancer cell proliferation is based on the interaction of a vertebrate estrogen receptor to a suite of compounds. Results of these studies do not necessarily allow the prediction of effects on invertebrates or potential bioaccumulation of environmental estrogens. It was important to collect a set of samples across a wide range of marine coastal environments so that effects of estrogens on biota at naturally occurring concentrations in seawater can be further characterized.

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